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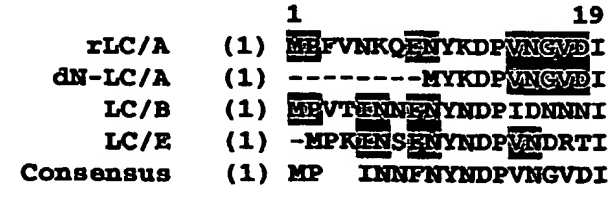
(54) Title: CHIMERA BOTULINUM TOXIN TYPE E



(57) Abstract: The present invention relates to a toxin comprising a modified light chain of a botulinum toxin type E, wherein the modified light chain comprises amino acid sequence PFVNKQFN (SEQ ID NO: 144) at the N-terminus, and amino acid sequence xBxxxLL (SEQ ID NO: 112) at the C-terminus, wherein x is any amino acid.

CHIMERA BOTULINUM TOXIN TYPE E

by

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CROSS REFERENCE

[0001] This application is a continuation-in-part of application serial number 10/757,077, filed January 14, 2004; which is a continuation-in-part of application serial number 10/163,106, filed June 4, 2002; which is a continuation-in-part of application serial number 09/910,346, filed July 20, 2001; which is a continuation-in-part of application serial number 09/620,840, filed July 21, 2000. All prior applications are incorporated herein by reference in their entireties.

BACKGROUND

[0002] The present invention relates to modified neurotoxins, particularly modified Clostridial neurotoxins, and use thereof to treat various conditions, including conditions that have been treated using naturally occurring botulinum toxins. For example, botulinum toxin type A has been used in the treatment of numerous conditions including pain, skeletal muscle conditions, smooth muscle conditions and glandular conditions. Botulinum toxins are also used for cosmetic purposes.

[0003] Numerous examples exist for treatment using botulinum toxin. For examples of treating pain see Aoki, et al., U.S. Patent 6,113,915 and Aoki, et al., U.S. Patent 5,721,215. For an example of treating a neuromuscular disorder, see U.S. Pat. No. 5,053,005, which suggests treating curvature of the juvenile spine, i.e., scoliosis, with an acetylcholine release inhibitor, preferably botulinum toxin A. For

the treatment of strabismus with botulinum toxin type A, see Elston, J. S., et al., British Journal of Ophthalmology, 1985, 69, 718-724 and 891-896. For the treatment of blepharospasm with botulinum toxin type A, see Adenis, J. P., et al., J. Fr. Ophthalmol., 1990, 13 (5) at pages 259-264. For treating spasmodic and oromandibular dystonia torticollis, see Jankovic et al., Neurology, 1987, 37, 616-623. Spasmodic dysphonia has also been treated with botulinum toxin type A. See Blitzer et al., Ann. Otol. Rhino. Laryngol, 1985, 94, 591-594. Lingual dystonia was treated with botulinum toxin type A according to Brin et al., Adv. Neurol. (1987) 50, 599-608. Cohen et al., Neurology (1987) 37 (Suppl. 1), 123-4, discloses the treatment of writer's cramp with botulinum toxin type A.

[0004] It would be beneficial to have botulinum toxins with enhanced biological persistence and/or enhanced biological activity.

SUMMARY

[0005] The present invention relates to a modified toxin comprising a modified light chain of a botulinum toxin type E, wherein the modified light chain comprises an amino acid sequence SEQ ID NO: 144 (PFVNKQFN) at the N-terminus, and an amino acid sequence SEQ ID NO: 112 (xExxxLL) at the C-terminus, wherein x is any amino acid.

DEFINITIONS

[0006] Before proceeding to describe the present invention, the following definitions are provided and apply herein.

[0007] "Heavy chain" means the heavy chain of a Clostridial neurotoxin. It has a molecular weight of about 100 kDa and can be referred to herein as Heavy chain or as H.

[0008] "H_N" means a fragment (having a molecular weight of about 50 kDa) derived from the Heavy chain of a Clostridial neurotoxin which is approximately

equivalent to the amino terminal segment of the Heavy chain, or the portion corresponding to that fragment in the intact Heavy chain. It is believed to contain the portion of the natural or wild-type Clostridial neurotoxin involved in the translocation of the light chain across an intracellular endosomal membrane.

[0009] "H_C" means a fragment (about 50 kDa) derived from the Heavy chain of a Clostridial neurotoxin which is approximately equivalent to the carboxyl terminal segment of the Heavy chain, or the portion corresponding to that fragment in the intact Heavy chain. It is believed to be immunogenic and to contain the portion of the natural or wild-type Clostridial neurotoxin involved in high affinity binding to various neurons (including motor neurons), and other types of target cells.

[0010] "Light chain" means the light chain of a Clostridial neurotoxin. It has a molecular weight of about 50 kDa, and can be referred to as light chain, L or as the proteolytic domain (amino acid sequence) of a Clostridial neurotoxin. The light chain is believed to be effective as an inhibitor of exocytosis, including as an inhibitor of neurotransmitter (i.e. acetylcholine) release when the light chain is present in the cytoplasm of a target cell.

[0011] "Neurotoxin" means a molecule that is capable of interfering with the functions of a cell, including a neuron. The "neurotoxin" can be naturally occurring or man-made. The interfered with function can be exocytosis.

[0012] "Modified neurotoxin" (or "modified toxin") means a neurotoxin which includes a structural modification. In other words, a "modified neurotoxin" is a neurotoxin which has been modified by a structural modification. The structural modification changes the biological persistence, such as the biological half-life (i.e. the duration of action of the neurotoxin) and/or the biological activity of the modified neurotoxin relative to the neurotoxin from which the modified neurotoxin is made or derived. The modified neurotoxin is structurally different from a naturally existing neurotoxin.

[0013] "Mutation" means a structural modification of a naturally occurring protein or nucleic acid sequence. For example, in the case of nucleic acid mutations, a mutation can be a deletion, addition or substitution of one or more nucleotides in the DNA sequence. In the case of a protein sequence mutation, the mutation can be a deletion, addition or substitution of one or more amino acids in a protein sequence. For example, a specific amino acid comprising a protein sequence can be substituted for another amino acid, for example, an amino acid selected from a group which includes the amino acids alanine, aspargine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine or any other natural or non-naturally occurring amino acid or chemically modified amino acids. Mutations to a protein sequence can be the result of mutations to DNA sequences that when transcribed, and the resulting mRNA translated, produce the mutated protein sequence. Mutations to a protein sequence can also be created by fusing a peptide sequence containing the desired mutation to a desired protein sequence.

[0014] "Structural modification" means any change to a neurotoxin that makes it physically or chemically different from an identical neurotoxin without the structural modification.

[0015] "Biological persistence" or "persistence" means the time duration of interference or influence caused by a neurotoxin or a modified neurotoxin with a cellular (such as a neuronal) function, including the temporal duration of an inhibition of exocytosis (such as exocytosis of neurotransmitter, for example, acetylcholine) from a cell, such as a neuron.

[0016] "Biological half-life" or "half-life" means the time that the concentration of a neurotoxin or a modified neurotoxin, preferably the active portion of the neurotoxin or modified neurotoxin, for example, the light chain of Clostridial

toxins, is reduced to half of the original concentration in a mammalian cell, such as in a mammalian neuron.

[0017] "Biological activity" or "activity" means the amount of cellular exocytosis inhibited from a cell per unit of time, such as exocytosis of a neurotransmitter from a neuron.

[0018] "Target cell" means a cell (including a neuron) with a binding affinity for a neurotoxin or for a modified neurotoxin.

[0019] "PURE A" means a purified botulinum toxin type A, that is the 150 kDa toxin molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **Figure 1:** Comparison of the N-terminal sequence of LC/A (Allergan Hall A), LC/B, and LC/E. dN-LC/A shows the amino acids truncated in our N-terminus deletion mutant.

[0021] **Figure 2:** Sequence comparison of the C-terminus of the Allergan Hall A LC/A with the C-terminus of different strains of LC/E. The box contains the di-leucine motif present on the LC/A. The sequence in that area is very well conserved in all the LC/Es and contains two Isoleucines instead of the Leucines.

[0022] **Figure 3:** LC/E chimeras generated by adding the localization signals of the LC/A into the LC/E. Constructs were generated by site-directed mutagenesis to incorporate a di-leucine motif at the C-terminus of the LC/E, and to add the N-terminus of the LC/A at the N-terminus of the LC/E.

[0023] **Figure 4:** Catalytic activity towards the cleavage of SNAP25 of the LC/E chimeras containing the localization signals from the LC/A and expressed in SH-SY5Y cells. Two separate transfections were performed and the western blot

data from both experiments are shown in the figure. Blots were probed with antibody SMI-81 to the N-terminus of SNAP25.

[0024] **Figure 5:** Plasmids encoding for the GFP-LC/E chimeras were transfected into PC-12 and SH-SY5Y cells. Three separate experiments were performed. Experiment number one is the top panel, experiment number two is the middle panel, and experiment number three is the bottom panel. Cell lysates were prepared and subjected to immunoprecipitation (top gels in each panel) with an antibody to GFP to detect the expressed protein. Part of the lysate was used for western blots to detect catalytic activity (bottom gels in each panel) of the chimeras expressed in cells. Each lane is numbered according to the table shown in the first panel, and reads as follow: 1. GFP negative control, 2. Wt LC/E, 3. LC/E with N-terminal LCA, 4. LC/E with C-terminal ExxxII, 5. LC/E with C-terminal ExxxLL, 6. LC/E with N-ter LCA and C-terminal ExxxII, 7. LC/E with N-ter LCA and C-terminal ExxxLL.

[0025] **Figure 6:** Taken from PNAS publication (1). Differentiated PC-12 cells expressing GFP-LC/A (A) and GFP-LC/E (B).

[0026] **Figure 7:** Differentiated PC-12 cells transfected with GFP-LCE (N-LCA/ExxxLL). Immunostained with antibodies to GFP, rabbit polyclonal at 1:100 (Figure 7A and 7C) and a combination of three Anti-$SNAP_{180}$ mouse monoclonal antibodies (1A3A7, 1G8C11 and 1C9F3), each at 1:50 dilution (Figure 7B and 7D). (63 x magnification).

[0027] **Figure 8:** Differentiated PC-12 cells transfected with GFP-LC/E (N-LCA/ExxxLL). Cells were immunostained with antibodies to GFP, mouse monoclonal at 1:100 (Figure 8A and 8C) and Anti-$SNAP25_{206}$ rabbit polyclonal antibody at 1:100 (Figure 8B and 8D). (63 x magnification). Specific cells are indicated by an arrow and are designated a, b, c, d or e. Transfected cells do not contain $SNAP25_{206}$ that is only present in non-transfected cells.

[0028] **Figure 9:** Differentiated PC-12 cells transfected with native beluga GFP-LC/E, and immunostained with rabbit polyclonal antibodies to GFP (Figure 9A) and 1:1:1 combination of anti-$SNAP25_{180}$ mouse monoclonal antibodies, each at 1:50 dilution (Figure 9B) (63 x magnification) as used in the previous figure with the chimeric LC/E. Both images correspond to the same cell but are not from the same plane.

[0029] **Figure 10:** SH-SY5Y cells transfected with GFP-LCE construct and stained with anti-GFP, rabbit polyclonal at a dilution of 1:100 and secondary anti-rabbit at 1:200. (63 x magnification). A and B represent two different cells from the same transfection experiment.

[0030] **Figure 11:** SH-SY5Y cells transfected with GFP-LCE (N-LCA/ExxxLL) and stained with anti-GFP antibodies. (63 x magnification). A and B are different groups of cells from the same transfection experiment.

[0031] **Figure 12:** SH-SY5Y cells transfected with GFP-LCE (N-LCA/ExxxLL). Cells were immunostained with antibodies to anti-GFP (Figures 12A and 12C) and uncleaved $SNAP25_{206}$ (Figure 12B & 12D). (63 x magnification).

[0032] **Figure 13:** SH-SY5Y cells transfected with GFP-LC/E (N-LCA/ExxxLL). Cells were immunostained with antibodies to GFP (Figure 13A) and 1A3A7 mouse monoclonal antibody specific for $SNAP25_{180}$ (Figure 13B). (63 x mag).

[0033] **Figure 14-a & b:** Sequence of wild-type Beluga LC/E. SEQ ID NO: 136 and SEQ ID NO: 137 correspond to the amino acid sequence and the nucleic acid sequence, respectively.

[0034] **Figure 15-a & b:** Sequence of chimera LC/E with N-terminus of LC/A. SEQ ID NO: 138 and SEQ ID NO: 139 correspond to the amino acid sequence and the nucleic acid sequence, respectively.

[0035] **Figure 16-a & b:** Sequence of chimera LC/E with LC/A di-leucine motif at C-terminus. SEQ ID NO: 140 and SEQ ID NO: 141 correspond to the amino acid sequence and the nucleic acid sequence, respectively.

[0036] **Figure 17-a & b:** Sequence of chimera LC/E with LC/A N-terminus and C-terminal di-leucine motif. SEQ ID NO: 142 and SEQ ID NO: 143 correspond to the amino acid sequence and the nucleic acid sequence, respectively.

DETAILED DESCRIPTION

[0037] The present invention is based upon the discovery that the biological persistence and/or the biological activity of a neurotoxin can be altered by structurally modifying the neurotoxin. In other words, a modified neurotoxin with an altered biological persistence and/or biological activity can be formed from a neurotoxin containing or including a structural modification. In some embodiments, the structural modification includes the fusing of a biological persistence enhancing component to the primary structure of a neurotoxin to enhance its biological persistence. In some embodiments, the biological persistence enhancing component is a leucine-based motif. Even more preferably, the biological half-life and/ or the biological activity of the modified neurotoxin is enhanced by about 100%. Generally speaking, the modified neurotoxin has a biological persistence of about 20% to 300% more than an identical neurotoxin without the structural modification. That is, for example, the modified neurotoxin including the biological persistence enhancing component is able to cause a substantial inhibition of neurotransmitter release for example, acetylcholine from a nerve terminal for about 20% to about 300% longer than a neurotoxin that is not modified.

[0038] The present invention also includes within its scope a modified neurotoxin with a biological activity altered as compared to the biological activity of the native

or unmodified neurotoxin. For example, the modified neurotoxin can exhibit a reduced or an enhanced inhibition of exocytosis (such as exocytosis of a neurotransmitter) from a target cell with or without any alteration in the biological persistence of the modified neurotoxin.

[0039] In a broad embodiment of the present invention, a leucine-based motif is a run of seven amino acids. The run is organized into two groups. The first five amino acids starting from the amino terminal of the leucine-based motif form a "quintet of amino acids." The two amino acids immediately following the quintet of amino acids form a "duplet of amino acids." In some embodiments, the duplet of amino acids is located at the carboxyl terminal region of the leucine-based motif. In some embodiments, the quintet of amino acids includes at least one acidic amino acid selected from a group consisting of a glutamate and an aspartate.

[0040] The duplet of amino acid includes at least one hydrophobic amino acid, for example leucine, isoleucine, methionine, alanine, phenylalanine, tryptophan, valine or tyrosine. Preferably, the duplet of amino acid is a leucine-leucine, a leucine-isoleucine, an isoleucine-leucine or an isoleucine-isoleucine, leucine-methionine. Even more preferably, the duplet is a leucine-leucine.

[0041] In some embodiments, the leucine-based motif is xDxxxLL, (SEQ ID NO:111) wherein x can be any amino acids. In some embodiments, the leucine-based motif is xExxxLL, (SEQ ID NO:112) wherein E is glutamic acid. In some embodiments, the duplet of amino acids can include an isoleucine or a methionine, forming xDxxxLI (SEQ ID NO:113) or xDxxxLM, (SEQ ID NO:114) respectively. Additionally, the aspartic acid, D, can be replaced by a glutamic acid, E, to form xExxxLI, (SEQ ID NO:115) xExxxIL (SEQ ID NO:116) and xExxxLM (SEQ ID NO:117). In some embodiments, the leucine-based motif is phenylalanine-glutamate-phenylalanine-tyrosine-lysine-leucine-leucine, SEQ ID NO:118.

[0042] In some embodiments, the quintet of amino acids comprises at least one hydroxyl containing amino acid, for example, a serine, a threonine or a tyrosine. Preferably, the hydroxyl containing amino acid can be phosphorylated. More preferably, the hydroxyl containing amino acid is a serine which can be phosphorylated to allow for the binding of adapter proteins.

[0043] Although non-modified amino acids are provided as examples, a modified amino acid is also contemplated to be within the scope of this invention. For example, leucine-based motif can include a halogenated, preferably, fluorinated leucine.

[0044] Various leucine-based motif are found in various species. A list of possible leucine-based motif derived from the various species that can be used in accordance with this invention is shown in Table 1. This list is not intended to be limiting.

[0045] TABLE 1

Species	Sequence	SEQ ID #
Botulinum type A	FEFYKLL	1
Rat VMAT1	EEKRAIL	2
Rat VMAT 2	EEKMAIL	3
Rat VAChT	SERDVLL	4
Rat δ	VDTQVLL	5
Mouse δ	AEVQALL	6
Frog γ/δ	SDKQNLL	7
Chicken γ/δ	SDRQNLI	8
Sheep δ	ADTQVLM	9
Human CD3γ	SDKQTLL	10
Human CD4	SQIKRLL	11
Human δ	ADTQALL	12
S. cerevisiae Vam3p	NEQSPLL	13

[0046] VMAT is vesicular monoamine transporter; VACht is vesicular acetylcholine transporter and S. cerevisiae Vam3p is a yeast homologue of synaptobrevin. Italicized serine residues are potential sites of phosphorylation.

[0047] The modified neurotoxin can be formed from any neurotoxin. Also, the modified neurotoxin can be formed from a fragment of a neurotoxin, for example, a botulinum toxin with a portion of the light chain and/or heavy chain removed. Preferably, the neurotoxin used is a Clostridial neurotoxin. A Clostridial neurotoxin comprises a polypeptide having three amino acid sequence regions. The first amino acid sequence region can include a target cell (i.e. a neuron) binding moiety which is substantially completely derived from a neurotoxin selected from a group consisting of baratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C_1, D, E, F, and G. Preferably, the first amino acid sequence region is derived from the carboxyl terminal region of a toxin heavy chain, H_C. Also, the first amino acid sequence region can comprise a targeting moiety which can comprise a molecule (such as an amino acid sequence) that can bind to a receptor, such as a cell surface protein or other biological component on a target cell.

[0048] The second amino acid sequence region is effective to translocate the polypeptide or a part thereof across an endosome membrane into the cytoplasm of a neuron. In some embodiments, the second amino acid sequence region of the polypeptide comprises an amine terminal of a heavy chain, H_N, derived from a neurotoxin selected from a group consisting of baratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C_1, D, E, F, and G.

[0049] The third amino acid sequence region has therapeutic activity when it is released into the cytoplasm of a target cell, such as a neuron. In some embodiments, the third amino acid sequence region of the polypeptide comprises a toxin light chain, L, derived from a neurotoxin selected from a group consisting of baratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C_1, D, E, F, and G.

[0050] The Clostridial neurotoxin can be a hybrid neurotoxin. For example, each of the neurotoxin's amino acid sequence regions can be derived from a different Clostridial neurotoxin serotype. For example, in one embodiment, the polypeptide comprises a first amino acid sequence region derived from the H_C of the tetanus toxin, a second amino acid sequence region derived from the H_N of botulinum type B, and a third amino acid sequence region derived from the light chain of botulinum serotype E. All other possible combinations are included within the scope of the present invention.

[0051] Alternatively, all three of the amino acid sequence regions of the Clostridial neurotoxin can be from the same species and same serotype. If all three amino acid sequence regions of the neurotoxin are from the same Clostridial neurotoxin species and serotype, the neurotoxin will be referred to by the species and serotype name. For example, a neurotoxin polypeptide can have its first, second and third amino acid sequence regions derived from Botulinum type E. In which case, the neurotoxin is referred as Botulinum type E.

[0052] Additionally, each of the three amino acid sequence regions can be modified from the naturally occurring sequence from which they are derived. For example, the amino acid sequence region can have at least one or more amino acids added or deleted as compared to the naturally occurring sequence.

[0053] A biological persistence enhancing component or a biological activity enhancing component, for example a leucine-based motif, can be fused with any of the above described neurotoxins to form a modified neurotoxin with an enhanced biological persistence and/or an enhanced biological activity. "Fusing" as used in the context of this invention includes covalently adding to or covalently inserting in between a primary structure of a neurotoxin. For example, a biological persistence enhancing component and/or a biological activity enhancing component can be added to a Clostridial neurotoxin which does not have a leucine-based motif in its

primary structure. In some embodiments, a leucine-based motif is fused with a hybrid neurotoxin, wherein the third amino acid sequence is derived from botulinum serotype A, B, C_1, C_2, D, E, F, or G. In some embodiments, the leucine-based motif is fused with a botulinum type E.

[0054] In some embodiments, a biological persistence enhancing component and/or a biological activity enhancing component is added to a neurotoxin by altering a cloned DNA sequence encoding the neurotoxin. For example, a DNA sequence encoding a biological persistence enhancing component and/or a biological activity enhancing component is added to a cloned DNA sequence encoding the neurotoxin into which the biological persistence enhancing component and/or a biological activity enhancing component is to be added. This can be done in a number of ways which are familiar to a molecular biologist of ordinary skill. For example, site directed mutagenesis or PCR cloning can be used to produce the desired change to the neurotoxin encoding DNA sequence. The DNA sequence can then be reintroduced into a native host strain. In the case of botulinum toxins the native host strain would be a *Clostridium botulinum* strain. Preferably, this host strain will be lacking the native botulinum toxin gene. In an alternative method, the altered DNA can be introduced into a heterologous host system such as *E. coli* or other prokaryotes, yeast, insect cell lines or mammalian cell lines. Once the altered DNA has been introduced into its host, the recombinant toxin containing the added biological persistence enhancing component and/or a biological activity enhancing component can be produced by, for example, standard fermentation methodologies.

[0055] Similarly, a biological persistence enhancing component can be removed from a neurotoxin. For example, site directed mutagenesis can be used to eliminate biological persistence enhancing components, for example, a leucine-based motif.

[0056] Standard molecular biology techniques that can be used to accomplish these and other genetic manipulations are found in Sambrook et al. (1989) which is incorporated in its entirety herein by reference.

[0057] In some embodiments, the leucine-based motif is fused with, or added to, the third amino acid sequence region of the neurotoxin. In some embodiments, the leucine-based motif is fused with, or added to, the region towards the carboxylic terminal of the third amino acid sequence region. More preferably, the leucine-based motif is fused with, or added to, the carboxylic terminal of the third region of a neurotoxin. Even more preferably, the leucine-based motif is fused with, or added to the carboxylic terminal of the third region of botulinum type E. The third amino acid sequence to which the leucine-based motif is fused or added can be a component of a hybrid or chimeric modified neurotoxin. For example, the leucine-based motif can be fused to or added to the third amino acid sequence region (or a part thereof) of one botulinum toxin type (i.e. a botulinum toxin type A), where the leucine-based motif-third amino acid sequence region has itself been fused to or conjugated to first and second amino acid sequence regions from another type (or types) of a botulinum toxin (such as botulinum toxin type B and/or E).

[0058] In some embodiments, a structural modification of a neurotoxin which has a pre-existing biological persistence enhancing component and/or a biological activity enhancing component, for example, a leucine-based motif includes deleting or substituting one or more amino acids of the leucine-based motif. In addition, a modified neurotoxin includes a structural modification which results in a neurotoxin with one or more amino acids deleted or substituted in the leucine-based motif. The removal or substitution of one or more amino acids from the preexisting leucine-based motif is effective to reduce the biological persistence and/or a biological activity of a modified neurotoxin. For example, the deletion or substitution of one or more amino acids of the leucine-based motif of botulinum type A reduces the biological half-life and/or the biological activity of the modified neurotoxin.

[0059] Amino acids that can be substituted for amino acids contained in a biological persistence enhancing component include alanine, aspargine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine,

methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine and other naturally occurring amino acids as well as non-standard amino acids.

[0060] In the present invention the native botulinum type A light chain has been shown to localize to differentiated PC12 cell membranes in a characteristic pattern. Biological persistence enhancing components are shown to substantially contribute to this localization.

[0061] The data of the present invention demonstrates that when the botulinum toxin type A light chain is truncated or when the leucine-based motif is mutated, the light chain substantially loses its ability to localize to the membrane in its characteristic pattern. Localization to the cellular membrane is believed to be a key factor in determining the biological persistence and/or the biological activity of a botulinum toxin. This is because localization to a cell membrane can protect the localized protein from intracellular protein degradation.

[0062] The deletion of the leucine-based motif from the light chain of botulinum type A can change membrane localization of the type A light chain. GFP fusion proteins were produced and visualized in differentiated PC12 cells using methods well known to those skilled in the art, for example, as described in Galli et al (1998) Mol Biol Cell 9:1437-1448, incorporated in its entirety herein by reference; also, for example, as described in Martinez-Arca et al (2000) J Cell Biol 149:889-899, also incorporated in its entirety herein by reference.

[0063] Further studies have been done in the present invention to analyze the effect of specific amino acid substitutions within the leucine-based motif. For example, in one study both leucine residues contained in the leucine-based motif were substituted for alanine residues. The substitution of alanine for leucine at positions 427 and 428 in the botulinum type A light chain substantially changes the localization characteristic of the light chain.

[0064] It is within the scope of this invention that a leucine-based motif, or any other persistence enhancing component and/or a biological activity enhancing component present on a light chain, can be used to protect the heavy chain as well. A random coil belt extends from the botulinum type A translocation domain and encircles the light chain. It is possible that this belt keeps the two subunits in proximity to each other inside the cell while the light chain is localized to the cell membrane.

[0065] In addition, the data of the present invention shows that the leucine-based motif can be valuable in localizing the botulinum A toxin in close proximity to the SNAP-25 substrate within the cell. This can mean that the leucine-based motif is important not only for determining the half-life of the toxin but for determining the activity of the toxin as well. That is, the toxin will have a greater activity if it is maintained in close proximity to the SNAP-25 substrate inside the cell. Dong et al., PNAS, 101(41): 14701-14706, 2004.

[0066] The data of the present invention clearly shows that truncation of the light chain, thereby deleting the leucine-based motif, or amino acid substitution within the leucine-based motif substantially changes membrane localization of the botulinum type A light chain in nerve cells. In both truncation and substitution a percentage of the altered light chain can localize to the cell membrane in a pattern unlike that of the native type A light chain. This data supports the presence of biological persistence enhancing components other than a leucine-based motif such as tyrosine motifs and amino acid derivatives. Use of these other biological persistence enhancing components and/or a biological activity enhancing components in modified neurotoxins is also within the scope of the present invention.

[0067] Also within the scope of the present invention is more than one biological persistence enhancing component used in combination in a modified neurotoxin to alter biological persistence of the neurotoxin that is modified. The present invention also includes use of more than one biological activity enhancing or biological

activity reducing components used in combination in a modified neurotoxin to alter the biological activity of the neurotoxin that is modified.

[0068] Tyrosine-based motifs are within the scope of the present invention as biological persistence and/or a biological activity altering components. Tyrosine-based motifs comprise the sequence Y-X-X-Hy (SEQ ID NO:119) where Y is tyrosine, X is any amino acid and Hy is a hydrophobic amino acid. Tyrosine-based motifs can act in a manner that is similar to that of leucine-based motifs.

[0069] Also within the scope of the present invention are modified neurotoxins which comprise one or more biological persistence altering components and/or a biological activity enhancing components which occur naturally in both botulinum toxin types A and B.

[0070] Amino acid derivatives are also within the scope of the present invention as biological persistence enhancing components and/or as biological activity enhancing components. Examples of amino acid derivatives that act to effect biological persistence and/or biological activity are phosphorylated amino acids. These amino acids include, for example, amino acids phosphorylated by tyrosine kinase, protein kinase C or casein kinase II. Other amino acid derivatives within the scope of the present invention as biological persistence enhancing components and/or as biological activity enhancing components are myristylated amino acids and N-glycosylated amino acids.

[0071] The present invention also contemplates compositions which include a botulinum light chain component interacting with a cellular structure component, for example, an intracellular structure component. The structure component may include lipid, carbohydrate, protein or nucleic acid or any combination thereof.

[0072] The structure component may include a cell membrane, for example, a plasma membrane. In certain embodiments, the structure component comprises all or part of one or more organelles, for example, the nucleus, endoplasmic reticulum,

golgi apparatus, mitochondria, lysosomes or secretory vesicles or combinations thereof. The structure component may include any portion of an organelle, for example, the membrane of an organelle. The structure component may also include any substance which is included in the cytoplasm of a cell.

[0073] The structure component may include one or more proteins. In some embodiments, the structure component includes one or more cellular proteins. One or more of these cellular proteins may be membrane associated proteins, for example, plasma membrane associated proteins. In some embodiments of the invention, the structure component includes adaptor proteins. Examples of adaptor proteins are AP-1, AP-2 and AP-3. Adaptor proteins and their characteristics are well known in the art and are discussed in, for example, Darsow et al., J. Cell Bio., 142, 913 (1998) which is incorporated in its entirety herein by reference. The one or more proteins may also include the substrate which is cleaved by the proteolytic domain of a botulinum toxin light chain component. For example, a protein included in the structure component may be SNAP-25.

[0074] The interaction between the light chain of botulinum type A and the structure component may contribute to localization of the toxin in a certain pattern. Therefore, the interaction may act to facilitate proteolysis by, for example, increasing the biological persistence and/or biological activity of the light chain.

[0075] A botulinum toxin heavy chain or portion thereof may also be associated with the light chain component when the light chain is interacting with the structure component.

[0076] In some embodiments, a botulinum toxin light chain component, when interacting with the structure component in a cell, may localize in the cell in a particular pattern. For example, localization of a botulinum toxin type A light chain component may be in a unctuate or spotted pattern. For example, a botulinum type A light chain component may be localized in a unctuate pattern on a cell

membrane, for example, a plasma membrane. Botulinum type B light chain may localize in the cytoplasm. Botulinum type E may localize to the plasma membrane but to a lesser degree than type A. Botulinum type E may also localize in the cytoplasm.

[0077] Methodologies to produce an isolated composition of the invention are available to those skilled in the art. For example, a composition may be isolated by isolating the plasma membrane from a cell after introduction of a light chain component, for example, light chain A, into a cell. The light chain may be introduced into the cell by, for example, electroporation or by endocytosis. In the case of introduction into the cell by endocytosis, a heavy chain component may be included with the light chain component to facilitate the endocytosis, for example, receptor mediated endocytosis, of the light chain. In such preparation process, the heavy chain component may also be isolated and be included in the composition.

[0078] After introduction into the cell, the light chain component associates or interacts with the substrate component forming a composition. The composition may be isolated by purification of the light chain component-structure component from the cell. Standard purification techniques known to those skilled in the art may be used to isolate a membrane and/or membrane associated protein(s) which is included in the structure component which interacts with the light chain component. Examples of conventional techniques for isolation and purification of the light chain component/structure component include immunoprecipitation and/or membrane purification techniques.

[0079] The light chain component may be crosslinked to a portion of the structure component before isolation. The technical procedures for cross linking of biomolecules using agents such as DTBP are well known to those skilled in the art.

[0080] In some embodiments, a composition of the invention may be prepared by mixing together a purified or a partially purified light chain component and a

purified or a partially purified intracellular structure component under conditions which are effective to form the composition. Conditions important in forming the composition may include Ph, ionic concentration and temperature.

[0081] The botulinum toxin light chain component of a composition, may be a modified botulinum toxin light chain. Modifications may be mutations and/or deletions as described elsewhere herein.

[0082] A modified light chain component may include a light chain A modified to remove a leucine based motif or other structure(s) which contributes to localization of the type A light chain to the plasma membrane thereby resulting in a light chain with a reduced ability to localize to a plasma membrane. This may result in a reduction in the biological activity and/or biological persistence of the light chain A. The biological persistence and/or activity of the modified light chain may be about 10% to about 90% that of an unmodified type A light chain.

[0083] Another modified light chain component may include a light chain A modified by adding one or more leucine based motifs, or other structure(s) which contributes to localization of the type A light chain to the plasma membrane, thereby resulting in a light chain with an increased ability to localize to a plasma membrane. This may result in an increase in the biological activity and/or biological persistence of the light chain A. The biological persistence and/or activity of the modified light chain may be about 1.5 to about 5 times that of an unmodified type A light chain.

[0084] A modified light chain component may include a light chain E modified by adding one or more leucine based motifs, or other structure(s) which contribute to localization of the type A light chain to the plasma membrane, thereby resulting in a light chain with an increased ability to localize to a plasma membrane. This may result in an increase in the biological activity and/or biological persistence of the light chain E. The biological persistence and/or activity of the modified light chain may be about 2 to about 20 times that of an unmodified type E light chain.

[0085] Compositions of the invention have many uses and applications, for example, in research science and medicine. Other uses and applications will be readily apparent to those skilled in the art.

[0086] In one broad aspect of the present invention, a method is provided for treating a condition using a modified neurotoxin. The conditions can include, for example, skeletal muscle conditions, smooth muscle conditions, pain and glandular conditions. The modified neurotoxin can also be used for cosmetics, for example, to treat brow furrows.

[0087] The neuromuscular disorders and conditions that can be treated with a modified neurotoxin include: for example, spasmodic dysphonia, laryngeal dystonia, oromandibular and lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorders, spasmodic torticolis, cerebral palsy, focal spasticity and other voice disorders, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia and other muscle tone disorders and other disorders characterized by involuntary movements of muscle groups can be treated using the present methods of administration. Other examples of conditions that can be treated using the present methods and compositions are lacrimation, hyperhydrosis, excessive salivation and excessive gastrointestinal secretions, as well as other secretory disorders. In addition, the present invention can be used to treat dermatological conditions, for example, reduction of brow furrows, reduction of skin wrinkles. The present invention can also be used in the treatment of sports injuries.

[0088] Borodic U.S. Patent No. 5,053,005 discloses methods for treating juvenile spinal curvature, i.e. scoliosis, using botulinum type A. The disclosure of Borodic is incorporated in its entirety herein by reference. In some embodiments, using substantially similar methods as disclosed by Borodic, a modified neurotoxin can be administered to a mammal, preferably a human, to treat spinal curvature. In some

embodiments, a modified neurotoxin comprising botulinum type E fused with a leucine-based motif is administered. Even more preferably, a modified neurotoxin comprising botulinum type A-E with a leucine-based motif fused to the carboxyl terminal of its light chain is administered to the mammal, preferably a human, to treat spinal curvature.

[0089] In addition, the modified neurotoxin can be administered to treat other neuromuscular disorders using well known techniques that are commonly performed with botulinum type A. For example, the present invention can be used to treat pain, for example, headache pain, pain from muscle spasms and various forms of inflammatory pain. For example, Aoki U.S. Patent NO: 5,721,215 and Aoki U.S. Patent No: 6,113,915 disclose methods of using botulinum toxin type A for treating pain. The disclosure of these two patents is incorporated in its entirety herein by reference.

[0090] Autonomic nervous system disorders can also be treated with a modified neurotoxin. For example, glandular malfunctioning is an autonomic nervous system disorder. Glandular malfunctioning includes excessive sweating and excessive salivation. Respiratory malfunctioning is another example of an autonomic nervous system disorder. Respiratory malfunctioning includes chronic obstructive pulmonary disease and asthma. Sanders et al. disclose methods for treating the autonomic nervous system; for example, treating autonomic nervous system disorders such as excessive sweating, excessive salivation, asthma, etc., using naturally existing botulinum toxins. The disclosure of Sander et al. is incorporated in its entirety by reference herein. In some embodiments, substantially similar methods to that of Sanders et al. can be employed, but using a modified neurotoxin, to treat autonomic nervous system disorders such as the ones discussed above. For example, a modified neurotoxin can be locally applied to the nasal cavity of the mammal in an amount sufficient to degenerate cholinergic neurons of the autonomic nervous system that control the mucous secretion in the nasal cavity.

[0091] Pain that can be treated by a modified neurotoxin includes pain caused by muscle tension, or spasm, or pain that is not associated with muscle spasm. For example, Binder in U.S. Patent No. 5,714,468 discloses that headache caused by vascular disturbances, muscular tension, neuralgia and neuropathy can be treated with a naturally occurring botulinum toxin, for example Botulinum type A. The disclosures of Binder are incorporated in its entirety herein by reference. In some embodiments, substantially similar methods to that of Binder can be employed, but using a modified neurotoxin, to treat headache, especially the ones caused by vascular disturbances, muscular tension, neuralgia and neuropathy. Pain caused by muscle spasm can also be treated by an administration of a modified neurotoxin. For example, a botulinum type E fused with a leucine-based motif, preferably at the carboxyl terminal of the botulinum type E light chain, can be administered intramuscularly at the pain/spasm location to alleviate pain.

[0092] Furthermore, a modified neurotoxin can be administered to a mammal to treat pain that is not associated with a muscular disorder, such as spasm. In one broad embodiment, methods of the present invention to treat non-spasm related pain include central administration or peripheral administration of the modified neurotoxin.

[0093] For example, Foster et al. in U.S. Patent No. 5,989,545 discloses that a botulinum toxin conjugated with a targeting moiety can be administered centrally (intrathecally) to alleviate pain. The disclosures of Foster et al. are incorporated in its entirety by reference herein. In some embodiments, substantially similar methods to that of Foster et al. can be employed, but using the modified neurotoxin according to this invention, to treat pain. The pain to be treated can be an acute pain, or preferably, chronic pain.

[0094] An acute or chronic pain that is not associated with a muscle spasm can also be alleviated with a local, peripheral administration of the modified neurotoxin to an actual or a perceived pain location on the mammal. In some embodiments, the

modified neurotoxin is administered subcutaneously at or near the location of pain, for example, at or near a cut. In some embodiments, the modified neurotoxin is administered intramuscularly at or near the location of pain, for example, at or near a bruise location on the mammal. In some embodiments, the modified neurotoxin is injected directly into a joint of a mammal, for treating or alleviating pain caused by arthritic conditions. Also, frequent repeated injection or infusion of the modified neurotoxin to a peripheral pain location is within the scope of the present invention. However, given the long lasting therapeutic effects of the present invention, frequent injection or infusion of the neurotoxin can not be necessary. For example, practice of the present invention can provide an analgesic effect, per injection, for 2 months or longer, for example 27 months, in humans.

[0095] Without wishing to limit the invention to any mechanism or theory of operation, it is believed that when the modified neurotoxin is administered locally to a peripheral location, it inhibits the release of Neuro-substances, for example substance P, from the peripheral primary sensory terminal by inhibiting SNARE-dependent exocytosis. Since the release of substance P by the peripheral primary sensory terminal can cause or at least amplify pain transmission process, inhibition of its release at the peripheral primary sensory terminal will dampen the transmission of pain signals from reaching the brain.

[0096] The amount of the modified neurotoxin administered can vary widely according to the particular disorder being treated, its severity and other various patient variables including size, weight, age, and responsiveness to therapy. Generally, the dose of modified neurotoxin to be administered will vary with the age, presenting condition and weight of the mammal, preferably a human, to be treated. The potency of the modified neurotoxin will also be considered.

[0097] Assuming a potency (for a botulinum toxin type A) which is substantially equivalent to LD_{50} = 2,730 U in a human patient and an average person

is 75kg, a lethal dose (for a botulinum toxin type A) would be about 36 U/kg of a modified neurotoxin. Therefore, when a modified neurotoxin with such an LD_{50} is administered, it would be appropriate to administer less than 36 U/kg of the modified neurotoxin into human subjects. Preferably, about 0.01 U/kg to 30 U/kg of the modified neurotoxin is administered. More preferably, about 1 U/kg to about 15 U/kg of the modified neurotoxin is administered. Even more preferably, about 5 U/kg to about 10 U/kg modified neurotoxin is administered. Generally, the modified neurotoxin will be administered as a composition at a dosage that is proportionally equivalent to about 2.5 cc/100 U. Those of ordinary skill in the art will know, or can readily ascertain, how to adjust these dosages for neurotoxin of greater or lesser potency. It is known that botulinum toxin type B can be administered at a level about fifty times higher that that used for a botulinum toxin type A for similar therapeutic effect. Thus, the units amounts set forth above can be multiplied by a factor of about fifty for a botulinum toxin type B.

[0098] Although examples of routes of administration and dosages are provided, the appropriate route of administration and dosage are generally determined on a case by case basis by the attending physician. Such determinations are routine to one of ordinary skill in the art (see for example, *Harrison's Principles of Internal Medicine* (1998), edited by Anthony Fauci et al., 14th edition, published by McGraw Hill). For example, the route and dosage for administration of a modified neurotoxin according to the present disclosed invention can be selected based upon criteria such as the solubility characteristics of the modified neurotoxin chosen as well as the types of disorder being treated.

[0099] The modified neurotoxin can be produced by chemically linking the leucine-based motif to a neurotoxin using conventional chemical methods well known in the art. For example, botulinum type E can be obtained by establishing and growing cultures of *Clostridium botulinum* in a fermenter, and then harvesting and purifying the fermented mixture in accordance with known procedures.

[00100] The modified neurotoxin can also be produced by recombinant techniques. Recombinant techniques are preferable for producing a neurotoxin having amino acid sequence regions from different Clostridial species or having modified amino acid sequence regions. Also, the recombinant technique is preferable in producing botulinum type A with the leucine-based motif being modified by deletion. The technique includes steps of obtaining genetic materials from natural sources, or synthetic sources, which have codes for a cellular binding moiety, an amino acid sequence effective to translocate the neurotoxin or a part thereof, and an amino acid sequence having therapeutic activity when released into a cytoplasm of a target cell, preferably a neuron. In some embodiments, the genetic materials have codes for the biological persistence enhancing component, preferably the leucine-based motif, the H_C, the H_N and the light chain of the Clostridial neurotoxins and fragments thereof. The genetic constructs are incorporated into host cells for amplification by first fusing the genetic constructs with a cloning vectors, such as phages or plasmids. Then the cloning vectors are inserted into a host, for example, *Clostridium* sp., *E. coli* or other prokaryotes, yeast, insect cell line or mammalian cell lines. Following the expressions of the recombinant genes in host cells, the resultant proteins can be isolated using conventional techniques.

[00101] There are many advantages to producing these modified neurotoxins recombinantly. For example, to form a modified neurotoxin, a modifying fragment, or component must be attached or inserted into a neurotoxin. The production of neurotoxin from anaerobic Clostridium cultures is a cumbersome and time-consuming process including a multi-step purification protocol involving several protein precipitation steps and either prolonged and repeated crystallization of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure must be performed under strict containment (BL-3). During the fermentation process, the folded single-chain neurotoxins are activated by endogenous Clostridial proteases through a process termed nicking to

create a dichain. Sometimes, the process of nicking involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the intrachain disulfide bond.

[00102] The nicked neurotoxin is much more active than the unnicked form. The amount and precise location of nicking varies with the serotypes of the bacteria producing the toxin. The differences in single-chain neurotoxin activation and, hence, the yield of nicked toxin, are due to variations in the serotype and amounts of proteolytic activity produced by a given strain. For example, greater than 99% of *Clostridial botulinum* serotype A single-chain neurotoxin is activated by the Hall A *Clostridial botulinum* strain, whereas serotype B and E strains produce toxins with lower amounts of activation (0 to 75% depending upon the fermentation time). Thus, the high toxicity of the mature neurotoxin plays a major part in the commercial manufacture of neurotoxins as therapeutic agents.

[00103] The degree of activation of engineered Clostridial toxins is, therefore, an important consideration for manufacture of these materials. It would be a major advantage if neurotoxins such as botulinum toxin and tetanus toxin could be expressed, recombinantly, in high yield in rapidly-growing bacteria (such as heterologous *E. coli* cells) as relatively non-toxic single-chains (or single chains having reduced toxic activity) which are safe, easy to isolate and simple to convert to the fully-active form.

[00104] With safety being a prime concern, previous work has concentrated on the expression in *E. coli* and purification of individual H and light chains of tetanus and botulinum toxins; these isolated chains are, by themselves, non-toxic; see Li et al., *Biochemistry* 33:7014-7020 (1994); Zhou et al., *Biochemistry* 34:15175-15181 (1995), hereby incorporated by reference herein. Following the separate production of these peptide chains and under strictly controlled conditions the H and

light chains can be combined by oxidative disulphide linkage to form the neuroparalytic di-chains.

EXAMPLES

[00105] The following non-limiting examples provide those of ordinary skill in the art with specific suitable methods to treat non-spasm related pain within the scope of the present invention and are not intended to limit the scope of the invention.

Example 1

[00106] Treatment of Pain Associated with Muscle Disorder:

[00107] An unfortunate 36 year old woman has a 15 year history of temporomandibular joint disease and chronic pain along the masseter and temporalis muscles. Fifteen years prior to evaluation she noted increased immobility of the jaw associated with pain and jaw opening and closing and tenderness along each side of her face. The left side is originally thought to be worse than the right. She is diagnosed as having temporomandibular joint (TMJ) dysfunction with subluxation of the joint and is treated with surgical orthoplasty meniscusectomy and condyle resection.

[00108] She continues to have difficulty with opening and closing her jaw after the surgical procedures and for this reason, several years later, a surgical procedure to replace prosthetic joints on both sides is performed. After the surgical procedure progressive spasms and deviation of the jaw ensues. Further surgical revision is performed subsequent to the original operation to correct prosthetic joint loosening. The jaw continues to exhibit considerable pain and immobility after these surgical procedures. The TMJ remained tender as well as the muscle itself. There are tender points over the temporomandibular joint as well as increased tone in the entire muscle. She is diagnosed as having post-surgical myofascial pain syndrome and is injected with the modified neurotoxin into the masseter and temporalis

muscles; the modified neurotoxin is botulinum type E comprising a leucine-based motif. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician.

[00109] Several days after the injections she noted substantial improvement in her pain and reports that her jaw feels looser. This gradually improves over a 2 to 3 week period in which she notes increased ability to open the jaw and diminishing pain. The patient states that the pain is better than at any time in the last 4 years. The improved condition persists for up to 27 months after the original injection of the modified neurotoxin.

Example 2

[00110] Treatment of Pain Subsequent to Spinal Cord Injury:

[00111] A patient, age 39, experiencing pain subsequent to spinal cord injury is treated by intrathecal administration, for example, by spinal tap or by catherization (for infusion) to the spinal cord, with the modified neurotoxin; the modified neurotoxin is botulinum type E comprising a leucine-based motif. The particular toxin dose and site of injection, as well as the frequency of toxin administrations, depend upon a variety of factors within the skill of the treating physician, as previously set forth. Within about 1 to about 7 days after the modified neurotoxin administration, the patient's pain is substantially reduced. The pain alleviation persists for up to 27 months.

Example 3

[00112] Peripheral Administration of a Modified Neurotoxin to Treat "Shoulder-Hand Syndrome":

[00113] Pain in the shoulder, arm, and hand can develop, with muscular dystrophy, osteoporosis and fixation of joints. While most common after coronary insufficiency, this syndrome can occur with cervical osteoarthritis or localized

shoulder disease, or after any prolonged illness that requires the patient to remain in bed.

[00114] A 46 year old woman presents a shoulder-hand syndrome type pain. The pain is particularly localized at the deltoid region. The patient is treated by a bolus injection of a modified neurotoxin subcutaneously to the shoulder; preferably the modified neurotoxin is botulinum type E comprising a leucine-based motif. The modified neurotoxin can also be, for example, modified botulinum type A, B, C1, C2, D, E, F or G which comprise a leucine-based motif. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician, as previously set forth. Within 1-7 days after modified neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

Example 4

[00115] Peripheral Administration of a Modified Neurotoxin to Treat Postherapeutic Neuralgia:

[00116] Postherapeutic neuralgia is one of the most intractable of chronic pain problems. Patients suffering this excruciatingly painful process often are elderly, have debilitating disease, and are not suitable for major interventional procedures. The diagnosis is readily made by the appearance of the healed lesions of herpes and by the patient's history. The pain is intense and emotionally distressing. Postherapeutic neuralgia can occur anywhere, but is most often in the thorax.

[00117] A 76 year old man presents a postherapeutic type pain. The pain is localized to the abdomen region. The patient is treated by a bolus injection of a modified neurotoxin intradermally to the abdomen; the modified neurotoxin is, for example, botulinum type A, B, C1, C2, D, E, F and/or G. The modified neurotoxin comprises a leucine-based motif and/or additional tyrosine-based motifs. The particular dose as well as the frequency of administration depends upon a variety of factors within the skill of the treating physician, as previously set forth. Within 1-7

days after modified neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

Example 5

[00118] Peripheral Administration of a Modified Neurotoxin to Treat Nasopharyngeal Tumor Pain:

[00119] These tumors, most often squamous cell carcinomas, are usually in the fossa of Rosenmuller and can invade the base of the skull. Pain in the face is common. It is constant, dull-aching in nature.

[00120] A 35 year old man presents a nasopharyngeal tumor type pain. Pain is found at the lower left cheek. The patient is treated by a bolus injection of a modified neurotoxin intramuscularly to the cheek, preferably the modified neurotoxin is botulinum type A, B, C1, C2, D, E, F or G comprising additional biological persistence enhancing amino acid derivatives, for example, tyrosine phosphorylations. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician. Within 1-7 days after modified neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

Example 6

[00121] Peripheral Administration of a Modified Neurotoxin to Treat Inflammatory Pain:

[00122] A patient, age 45, presents an inflammatory pain in the chest region. The patient is treated by a bolus injection of a modified neurotoxin intramuscularly to the chest, preferably the modified neurotoxin is botulinum type A, B, C1, C2, D, E, F or G comprising additional tyrosine-based motifs. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician, as previously set forth. Within 1-7 days after modified

neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

Example 7

[00123] Treatment of Excessive Sweating:

[00124] A male, age 65, with excessive unilateral sweating is treated by administering a modified neurotoxin. The dose and frequency of administration depends upon degree of desired effect. Preferably, the modified neurotoxin is botulinum type A, B, C1, C2, D, E, F and/or G. The modified neurotoxins comprise a leucine-based motif. The administration is to the gland nerve plexus, ganglion, spinal cord or central nervous system. The specific site of administration is to be determined by the physician's knowledge of the anatomy and physiology of the target glands and secretory cells. In addition, the appropriate spinal cord level or brain area can be injected with the toxin. The cessation of excessive sweating after the modified neurotoxin treatment is up to 27 months.

Example 8

[00125] Post Surgical Treatments:

[00126] A female, age 22, presents a torn shoulder tendon and undergoes orthopedic surgery to repair the tendon. After the surgery, the patient is administered intramuscularly with a modified neurotoxin to the shoulder. The modified neurotoxin can botulinum type A, B, C, D, E, F, and/or G wherein one or more amino acids of a biological persistence enhancing component are deleted from the toxin. For example, one or more leucine residues can be deleted from and/or mutated from the leucine-based motif in botulinum toxin serotype A. Alternatively, one or more amino acids of the leucine-based motif can be substituted for other amino acids. For example, the two leucines in the leucine-based motif can be substituted for alanines. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating

physician. The specific site of administration is to be determined by the physician's knowledge of the anatomy and physiology of the muscles. The administered modified neurotoxin reduces movement of the arm to facilitate the recovery from the surgery. The effect of the modified neurotoxin is for about five weeks or less.

Example 9

[00127] Cloning, Expression and Purification of the Botulinum Neurotoxin Light Chain Gene:

[00128] This example describes methods to clone and express a DNA nucleotide sequence encoding a botulinum toxin light chain and purify the resulting protein product. A DNA sequence encoding the botulinum toxin light chain can be amplified by PCR protocols which employ synthetic oligonucleotides having sequences corresponding to the 5' and 3' end regions of the light chain gene. Design of the primers can allow for the introduction of restriction sites, for example, Stu I and EcoR I restriction sites into the 5' and 3' ends of the botulinum toxin light chain gene PCR product. These restriction sites can be subsequently used to facilitate unidirectional subcloning of the amplification products. Additionally, these primers can introduce a stop codon at the C-terminus of the light chain coding sequence. Chromosomal DNA from C. *botulinum*, for example, strain HallA, can serve as a template in the amplification reaction.

[00129] The PCR amplification can be performed in a 0.1 mL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 50 pmol of each primer, 200 ng of genomic DNA and 2.5 units of Taq DNA polymerase. The reaction mixture can be subjected to 35 cycles of denaturation (1 minute at 94° C), annealing (2 minutes at 55°C) and polymerization (2 minutes at 72°C). Finally, the reaction can be extended for an additional 5 minutes at 72°C.

[00130] The PCR amplification product can be digested with for example, Stu I and EcoR I, to release the light chain encoding, cloned, PCR DNA fragment. This fragment can then be purified by, for example, agarose gel electrophoresis, and

ligated into, for example, a Sma I and EcoR I digested pBluescript II SK phagemid. Bacterial transformants, for example, *E. coli*, harboring this recombinant phagemid can be identified by standard procedures, such as blue/white screening. Clones comprising the light chain encoding DNA can be identified by DNA sequence analysis performed by standard methods. The cloned sequences can be confirmed by comparing the cloned sequences to published sequences for botulinum light chains, for example, Binz, et al., in *J. Biol. Chem.* 265, 9153 (1990), Thompson et al., in *Eur. J. Biochem.* 189, 73 (1990) and Minton, Clostridial Neurotoxins, The Molecular Pathogenesis of Tetanus and Botulism p. 161-191, Edited by C. Motecucco (1995).

[00131] The light chain can be subcloned into an expression vector, for example, pMal-P2. pMal-P2 harbors the malE gene encoding MBP (maltose binding protein) which is controlled by a strongly inducible promoter, P_{tac}.

[00132] To verify expression of the botulinum toxin light chain, a well isolated bacterial colony harboring the light chain gene containing pMal-P2 can be used to inoculate L-broth containing 0.1 mg/ml ampicillin and 2% (w/v) glucose, and grown overnight with shaking at 30°C. The overnight cultures can be diluted 1:10 into fresh L-broth containing 0.1 mg/ml of ampicillin and incubated for 2 hours. Fusion protein expression can be induced by addition of IPTG to a final concentration of O.1 mM. After an additional 4 hour incubation at 30°C, bacteria can be collected by centrifugation at 6,000 x g for 10 minutes.

[00133] A small-scale SDS-PAGE analysis can confirm the presence of a 90 kDa protein band in samples derived from IPTG-induced bacteria. This MW would be consistent with the predicted size of a fusion protein having MBP (~ 40 kDa) and botulinum toxin light chain (~ 50 kDa) components.

[00134] The presence of the desired fusion proteins in IPTG-induced bacterial extracts can be confirmed by western blotting using the polyclonal anti-L chain probe described by Cenci di Bello et al., in *Eur. J. Biochem.* 219, 161 (1993). Reactive bands on PVDF membranes (Pharmacia; Milton Keynes, UK) can be visualized using an anti-rabbit immunoglobulin conjugated to horseradish peroxidase

(BioRad; Hemel Hempstead, UK) and the ECL detection system (Amersham, UK). Western blotting results typically confirm the presence of the dominant fusion protein together with several faint bands corresponding to proteins of lower MW than the fully sized fusion protein. This observation suggests that limited degradation of the fusion protein occurred in the bacteria or during the isolation procedure.

[00135] To produce the subcloned light chain, pellets from 1 liter cultures of bacteria expressing the wild-type Botulinum neurotoxin light chain proteins can be resuspended in column buffer [10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EGTA and 1 mM DTT] containing 1mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM benzamidine, and lysed by sonication. The lysates can be cleared by centrifugation at 15,000 x g for 15 minutes at 4°C. Supernatants can be applied to an amylose affinity column [2x10 cm, 30 ml resin] (New England BioLabs; Hitchin, UK). Unbound proteins can be washed from the resin with column buffer until the eluate is free of protein as judged by a stable absorbance reading at 280 nm. The bound MBP-L chain fusion protein can be subsequently eluted with column buffer containing 10 mM maltose. Fractions containing the fusion protein can be pooled and dialyzed against 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 2 mM, CaCl2 and 1 mM DTT for 72 hours at 4°C.

[00136] The MBP-L chain fusion proteins can be purified after release from the host bacteria. Release from the bacteria can be accomplished by enzymatically degrading or mechanically disrupting the bacterial cell membrane. Amylose affinity chromatography can be used for purification. Recombinant wild-type or mutant light chains can be separated from the sugar binding domains of the fusion proteins by site-specific cleavage with Factor Xa. This cleavage procedure typically yields free MBP, free light chains and a small amount of uncleaved fusion protein. While the resulting light chains present in such mixtures can be shown to possess the desired activities, an additional purification step can be employed. For example, the mixture of cleavage products can be applied to a second amylose affinity column which binds both the MBP and uncleaved fusion protein. Free light chains can be isolated in the flow through fraction.

Example 10

[00137] Reconstitution of Native light chain, Recombinant Wild-Type light chain with Purified Heavy chain:

[00138] Native heavy and light chains can be dissociated from a BoNT with 2 M urea, reduced with 100 mM DTT and then purified according to established chromatographic procedures. For example, Kozaki et al. (1981, *Japan J. Med. Sci. Biol.* 34, 61) and Maisey et al. (1988, *Eur. J. Biochem.* 177, 683). A purified heavy chain can be combined with an equimolar amount of either native light chain or a recombinant light chain. Reconstitution can be carried out by dialyzing the samples against a buffer consisting of 25 mM Tris (pH 8.0), 50 μM zinc acetate and 150 mM NaCl over 4 days at 4°C. Following dialysis, the association of the recombinant light chain and native heavy chain to form disulfide linked 150 kDa dichains is monitored by SDS-PAGE and/or quantified by densitometric scanning.

Example 11

[00139] Production of a Modified Neurotoxin with an Enhanced Biological Persistence:

[00140] A modified neurotoxin can be produced by employing recombinant techniques in conjunction with conventional chemical techniques.

[00141] A neurotoxin chain, for example a botulinum light chain that is to be fused with a biological persistence enhancing component to form a modified neurotoxin can be produced recombinantly and purified as described in example 9.

[00142] The recombinant neurotoxin chain derived from the recombinant techniques can be covalently fused with (or coupled to) a biological persistence enhancing component, for example a leucine-based motif, a tyrosine-based motif and/or an amino acid derivative. Peptide sequences comprising biological persistence enhancing components can be synthesized by standard t-Boc/Fmoc technologies in solution or solid phase as is known to those skilled in the art. Similar synthesis techniques are also covered by the scope of this invention, for example, methodologies employed in Milton et al. (1992, *Biochemistry* 31, 8799-

8809) and Swain et al. (1993, *Peptide Research* 6, 147-154). One or more synthesized biological persistence enhancing components can be fused to the light chain of botulinum type A, B, C1, C2, D, E, F or G at, for example, the carboxyl terminal end of the toxin. The fusion of the biological persistence enhancing components is achieved by chemical coupling using reagents and techniques known to those skilled in the art, for example PDPH/EDAC and Traut's reagent chemistry.

[00143] Alternatively, a modified neurotoxin can be produced recombinantly without the step of fusing the biological persistence enhancing component to a recombinant botulinum toxin chain. For example, a recombinant neurotoxin chain, for example, a botulinum light chain, derived from the recombinant techniques of example 9 can be produced with a biological persistence enhancing component, for example a leucine-based motif, a tyrosine-based motif and/or an amino acid derivative. For example, one or more DNA sequences encoding biological persistence enhancing components can be added to the DNA sequence encoding the light chain of botulinum type A, B, C1, C2, D, E, F or G. This addition can be done by any number of methods used for site directed mutagenesis which are familiar to those skilled in the art.

[00144] The recombinant modified light chain containing the fused or added biological persistence enhancing component can be reconstituted with a heavy chain of a neurotoxin by the method described in example 10 thereby producing a complete modified neurotoxin.

[00145] The modified neurotoxins produced according to this example have an enhanced biological persistence. Preferably, the biological persistence is enhanced by about 20% to about 300% relative to an identical neurotoxin without the additional biological persistence enhancing component(s).

Example 12

[00146] The first 30 residues of the amino-terminus (N-term) and the last 50 residues of the carboxyl-terminal (C-term) of the amino acid sequences of botulinum toxin serotypes A through G light chains (LC) are shown in Table 2.

Table 2

toxin	N-term (AAs 1-30) of LC	C-term (last 50 AAs) of LC	Seq ID #s
BoNT/A	MPFVNKQFNYKDPVN GVDIAYIKIPNAGQM	GFNLRNTNLAANFNGQNTEINNMNF TKLKNFTGLFEFYKLLCVRGIITSK	14/15
BoNT/B	MPVTINNFNYNDPIDN DNIIMMEPPFARGT	YTIEEGFNISDKNMGKEYRGQNKAI NKQAYEEISKEHLAVYKIQMCKSVK	16/17
BoNT/C1	MPITINNFNYSDPVDN KNILYLDTHLNTLA	NIPKSNLNVLFMGQNLSRNPALRKV NPENMLYLFTKFCHKAIDGRSLYNK	18/19
BoNT/D	MTWPVKDFNYSDPVN DNDILYLRIPQNKLI	YTIRDGFNLTNKGFNIENSGQNIERN PALQKLSSESVVDLFTKVCLRLTK	20/21
BoNT/E	MPKINSFNYNDPVNDR TILYIKPGGCQEFY	GYNINNLKVNFRGQNANLNPRIITPIT GRGLVKKIIRFCKNIVSVKGIRK	22/23
BoNT/F	MPVAINSFNYNDPVN DDTILYMQIPYEEKS	TVSEGFNIGNLAVNNRGQSIKLNPKII DSIPDKGLVEKIVKFCKSVIPRK	24/25
BoNT/G	MPVNIKXFNYNDPINN DDIIMMEPFNDPGP	QNEGFNIASKNLKTEFNGQNKAVNK EAYEEISLEHLVIYRIAMCKPVMYK	26/27--

[00147] Alterations in the amino acid sequence of these serotypes can include amino acid substitutions, mutations, deletions, or various combinations of these alterations. Such alterations can be engineered in the first thirty amino acids (AAs) in the N-terminus of the light chain and/or the last fifty AAs in the C-terminus of the light chain using recombinant DNA technological methods that are standard in the art.

[00148] For example, studies showed that a GFP-LCA construct with eight amino acid residues (PFVNKQFN) (SEQ ID NO:120) deleted from the N-terminus (no C-terminus deletion) localized in PC12 cells a very similar pattern to the localization in PC12 cells of a truncated GFP-LCA construct with both the C and N terminus deletions.

[00149] Further studies showed that a GFP-LCA construct with twenty two amino acid residues (KNFTG LFEFYKLLCV RGIITSK) (SEQ ID NO:121) deleted

from the C-terminus (no N-terminus deletion) localized in PC12 cells in a very similar manner to that of the GFP-LCA(LL-->AA) mutant.

[00150] A GFP-LCA construct with both eight amino acid residues (PFVNKQFN) (SEQ ID NO:122) deleted from the N-terminus and twenty two amino acid residues (KNFTG LFEFYKLLCV RGIITSK) (SEQ ID NO:123) deleted from the C-terminus accumulated intracellularly.

[00151] Examples of amino acid sequence substitutions include the replacement of one or more contiguous or non-contiguous amino acids in the first 30 amino acids of the N-terminus and/or the last 50 amino acids of the C-terminus of the light chain with an equal number and placement of amino acids that differ from the wild-type sequence. Substitutions can be conservative or non-conservative of the character of the amino acid. For example, the amino acid valine at a specific position in the wild-type sequence can be replaced with an alanine in the same position in the substituted sequence. Furthermore, basic residues such as arginine or lysine can be substituted for highly hydrophobic residues such as tryptophan. A proline or histidine residue may be substituted in order to form or disrupt a potentially important structural or catalytic element of the protein. Some examples of amino acid substitutions are indicated by bold underlined text in the sequences described in Table 3.

Table 3

toxin	N-term (AAs 1-30) of LC	C-term (last 50 AAs) of LC	Seq ID #
BoNT/A	MPF**A**NKQFNYKDPVN GVDIAYIKIPNAGQM	GFNLRNTNLAANFNGQNTEINNM N**R**TKLKNFTGLFEFYKLLCVRGIIT SK	28/29
BoNT/A	MPFVNKQFN**K**KDPVN GVDIAYIKIPNAGQM	GFNLRNTNLAANFNGQNTEINNM NFTKLKN**AA**GLFEFYKLLCVRGIIT SK	30/31
BoNT/A	MPFVNKQFNYKDPVN GVDIA**R**IKIPNAGQM	GFNLRNTNLAAN**H**NGQNTEINNM NFTKLKNFTGLFEFYKLLCVRGIIT SK	32/33

BoNT/A	MPFVNKHFNYKDPVN GVDIAYIKIPNAGQM	GFNLRNTNLAANFNGQNTEINNM NFTKLKNFTGLFEFYKLLCARGIIT SK	34/35
BoNT/B	MPATINNFNYNDPIDN DNIIMMEPPFARGT	YTIEEGFNISDKNMGKEYRGQNKA INKQAYEEISKEHLAVYKIRMCKS VK	36/37
BoNT/B	MPVTINNFNYNDPIDN DNIIAAEPPFARGT	YTIEEGFNISDKNMGKEYRGQNKA INKQAYEEISKEHLAVRKIQMCKS VK	38/39
BoNT/B	MPVTINNFNRNDPIDN DNIIMMEPPFARGT	YTIEEGFNISDKNMGKEYRGQNKA INKQAKEEISKEHLAVYKIQMCKS VK	40/41
BoNT/C1	MPITINNKNYSDPVDN KNILYLDTHLNTLA	NIPKSNLNVLFMGQNLSRNPALRK VNPENMLYLFTKFCHKAIDGRSLR NK	42/43
BoNT/D	MTWPAKDFNYSDPAN DNDILYLRIPQNKLI	YTIRDGFNLTNKGFNIENSGQNIER NPALQKLSSESVVDLFTKACLRLT K	44/45
BoNT/E	MPKINSFNYNDPANDR TILYIKPGGCQEFY	GYNINNLKVNFRGQNANLNPRIITP ITGRGHVKKIIRFCKNIVSVKGIRK	46/47
BoNT/E	MPKINSRNYNDPVND RTILYIKPGGCQEFY	GYNINNLKVNFRGQNANLNPRIITP ITGRGLVKKIIRFCKNAASVKGIRK	48/49
BoNT/E	MPKINSFNYNDPVNDR TILYIKPGGCQEFR	GYNINNLKVNFRGQNANLNPRIITP ITGRGLVKKIIRFCKNIVSAKGIRK	50/51
BoNT/F	MPAAINSFNYNDPVN DDTILYMQIPYEEKS	TVSEGFNIGNLAVNNRGQSIKLNP KIIDSIPDKGLVEKIVKFCKSAIPRK	52/53
BoNT/G	MPVNIKXHNYNDPIN NDDIIMMEPFNDPGP	QNEGFNIASKNLKTEFNGQNKAVN KEAYEEISLEHLVIYRIAMCKPAM YK	54/55

[00152] Examples of amino acid sequence mutations include changes in the first 30 amino acids of the N-terminus and/or the last 50 amino acids of the C-terminus of the light chain sequence such that one or several amino acids are added,

substituted and/or deleted such that the identity, number and position of amino acids in the wild-type light chain sequence is not necessarily conserved in the mutated light chain sequence. Some examples of amino acid sequence mutations are described in Table 4, in which additions of amino acids are shown in bold underlined text, and deletions are indicated by dashes in the sequences shown.

Table 4

toxin	N-term (AAs 1-30) of LC	C-term (last 50 AAs) of LC	Seq ID #
BoNT/A	MPFVNKQFNYKDPVN GVDIAYIKIPH---	GFNLRNTNLAANFNGQNTEINNM NAAAAAAAAAA-----CVRGIITSK	56/57
BoNT/A	MAAA---- NYKDPVNGVDIAYIKI PNAGQM	GKNLRNTNLAANFNGQNTEINNM NFTKLKNFTGLFEFYK— CVRGIITSK	58/59
BoNT/A	MPFVNKQFNYKDPVN GVDIAR—NAGQM	GFNLRNTNLAA— HNTEINNMNFTKLKNFTGLFEFYK LLCVRGIITSK	60/61
BoNT/A	MPKVNKQFN---- VNGVDIAYIKIPNAGQ M	GFNLRNTNLAANFNGQNTEINNM NFTKLKNFTGLFEFRR------TSK	62/63
BoNT/B	MPVTINNFNYNDPIDN DNIIAAAAAAAARGT	YTIPPGFNISDKNMGKEYRGQNKA INKQAYEEISKEH----------	64/65
BoNT/B	MPA---- FNYNDPIDNDNIIMME PPFARGT	YTIEEGFNISDKNMGKEYRGQNKA AAAAAAEEISKEHLAVYKIQMCKS VK	66/67
BoNT/B	MPVTINNFNR-------- MMEPPFARGT	YTIEEGFNISDKNMGKEYRGQNKA INKQAY-----AAAAAAIQMCKSVK	68/69
BoNT/C1	M-------- SDPVDNKNILYLDTHL NTLA	NIPKSNLNVLFMGQNLSRNPALRK VNPENMLAAA— CHKAIDGRSLYNK	70/71
BoNT/D	MTRPVKD---- DPVNDNDILYLRIPQN KLI	YTIRDGFNLTNKGFNIENSGQNIER NPALQKL-----DLPPKVCLRLTK	72/73

BoNT/E	MPKINSPPNYNDPVND RTILYIKPGGCQEFY	GYNINNLKVNFRGQNANLNPRIITP ITGRGLVKKAAAACKNIVSVKGIR K	74/75
BoNT/E	MPKINSFNYNDPAAA ANDRTILYIKPGGCQE FY	GYNINNLKVNFRGQNANLNPRIITP ITGRGLV---HRFCKNIVSVKGIRK	76/77
BoNT/E	MPKINSFNYNDPVNDR TILKIKPGGCKEFY	GYNINNLKVNFRGQNANLNPRIITP ITGRGLPP----------	78/79
BoNT/F	MP------ NYNDPVNDDTILYMQI PYEEKS	TVSEGFNIGNLAVNNRGQSIKLNP KIIDSIPDKGAAAAAA--CKSVIPRK	80/81
BoNT/G	MPVNIPP--- DPINNDDIIMMEPFND PGP	QNEGFNIASKNLKTEFNGQNKAVN KEAY----------AAAAAAA	82/83

[00153] Examples of amino acid sequence deletions include the removal of one or more contiguous or non-contiguous amino acids from the first 30 amino acids of the N-terminus and/or the last 50 amino acids of the C-terminus of the light chain sequence. Some examples of amino acid sequence deletions are indicated by dashes in the sequences shown in Table 5.

Table 5

toxin	N-term (AAs 1-30) of LC	C-term (last 50 AAs) of LC	Seq ID #
BoNT/A	M------- YKDPVNGVDIAYIKIP NAGQM	GFNLRNTNLAANFNGQNTEINNMNFT KLKNFTGLFEFYK----------	84/85
BoNT/A	MPFVNKQ------ VNGVDIAYIKIPNAGQ M	GFNLRNTNLAANFNGQNTEINNMNFT KLK---------LLCVRGIITSK	86/87
BoNT/A	MPFVNKQFNYKDP---- -AYIKIPNAGQM	GFNLRNTNLAANFNGQNTEINNMN---- ----GLFEFYKLLCVRGIITSK	88/89
BoNT/A	MPFVNKQFNYKDPVN	GFNLRN----------	90/91

	GVDIA--------	NTEINNMNFTKLKNFTGLFEFYKLLCV RGIITSK	
BoNT/B	MPVTINNFNYNDPIDN DNIIMME------	YTI---- ISDKNMGKEYRGQNKAINKQAYEEISK EHLAVYKIQMCKSVK	92/93
BoNT/B	MPVTINNFNYND------ ---EPPFARGT	YTIEEGFNISD------- GQNKAINKQAYEEISKEHLAVYKIQM CKSVK	94/95
BoNT/B	MP------- NDPIDNDNIIMMEPPF ARGT	YTIEEGFNISDKNMGKEYRGQNKAINK QA----------KIQMCKSVK	96/97
BoNT/C1	MPI------ SDPVDNKNILYLDTHL NTLA	NIPKSNLNVLFMGQNLSRNPALRKV---- ------KFCHKAIDGRSLYNK	98/99
BoNT/D	MTW-------- VNDNDILYLRIPQNKLI	YTIRDGFNLTNKGFNIENSGQNIERNPA ---------DLFTKVCLRLTK	100/101
BoNT/E	MP------- DPVNDRTILYIKPGGC QEFY	GYNINNLKVNFRGQNANLNPRIITPI---- ------RFCKNIVSVKGIRK	102/103
BoNT/E	MPKINSFNYN--------- IKPGGCQEFY	GYNINN------ GQNANLNPRIITPITGRGLVKKIIRFCK NIVSVKGIRK	104/105
BoNT/E	MPKINSFNYNDPVNDR TILYIK--------	GYNINNLKVNFRGQNANLNPRIITPITG RGLVKKIIR-------KGIRK	106/107
BoNT/F	MPVAINSFNYNDPVN DDTILYMQIP-----	TVSEGFNIGNLAVNNRGQSIKLNPKIID SIPD--------KFCKSVIPRK	108/109
BoNT/G	M----------	QNEGFNIASKNLKTEFNGQNKAVNKE A----------RIAMCKPVMYK	110

Example 13

[00154] In some embodiments of the present invention, the biological

persistence and/or the enzymatic activity of a toxin can be altered by structurally modifying the toxin. In some embodiments, the structural modification includes the substitution, mutation or deletion of amino acids within the toxin. In some embodiments, the structural modification includes a chimeric fusion construct in which a biological persistence-enhancing component or an enzymatic activity-enhancing component may be fused to, swapped for, or incorporated within a terminal end of the light chain of a botulinum toxin. In some embodiments, the structural modification includes a chimeric fusion construct in which a biological persistence-reducing component or an enzymatic activity-reducing component may be fused to, swapped for, or incorporated within a terminal end of the light chain of a botulinum toxin. In some embodiments, the persistence- or activity-enhancing or persistence- or activity-reducing component is an N-terminal region including the first 30 amino acids of a light chain of a botulinum toxin, or a C-terminal region including the last 50 amino acids of a light chain of a botulinum toxin. This biological persistence- or enzymatic activity-enhancing component or biological persistence- or enzymatic activity-reducing component is swapped for, fused to, or incorporated within an N- and/or C-terminus of a light chain of a botulinum toxin to enhance or reduce its biological persistence and/or enzymatic activity.

[00155] In some embodiments, the fusion of, addition to, or swapping of the N-terminal region of the light chain of BoNT/A into a chimeric construct results in an increase in biological persistance and/or enzymatic activity. In some embodiments, a substituted, mutated, or deleted N-terminal region of the light chain of BoNT/A within a chimeric construct results in a decrease in biological persistance and/or enzymatic activity. In some embodiments, the fusion of, addition to, or swapping of the C-terminal region of the light chain of BoNT/A into a chimeric construct results in an increase in biological persistance and/or enzymatic activity. In some embodiments, a substituted, mutated, or deleted C-terminal region of the light chain of BoNT/A within a chimeric construct results in a decrease in biological persistance and/or enzymatic activity.

[00156] Generally, it is suitable that the chimeric toxin has a biological persistence of about 20% to 300% greater than an identical toxin without the

structural modification. The biological persistence of the chimeric toxin may be enhanced by about 100%. That is, for example, the modified botulinum neurotoxin including the biological persistence-enhancing component is able to cause a substantial inhibition of neurotransmitter release (for example, acetylcholine) from a nerve terminal for about 20% to about 300% longer than a neurotoxin without the structural modification.

[00157] Similarly, it is suitable that the chimeric botulinum toxin light chain has an altered enzymatic activity. For example, the chimeric toxin can exhibit a reduced or an enhanced inhibition of exocytosis (such as exocytosis of a neurotransmitter) from a target cell with or without any alteration in the biological persistence of the modified neurotoxin. Altered enzymatic activities include increased or decreased efficiency or potency, increased or decreased localization to the plasma membrane, increased or decreased substrate specificity, and/or increased or decreased rate of proteolysis of SNAP/SNARE proteins. An increase in enzymatic activity can be from 1.5 to 5 times greater than the biological activity of the native or unmodified light chain. For example, the chimeric botulinum neurotoxin including the enzymatic activity-enhancing component is able to cause a substantial inhibition of neurotransmitter release (for example, acetylcholine) from a nerve terminal due to an increased rate of proteolysis of the SNAP-25 substrate as compared to a neurotoxin without the structural modification.

[00158] It has been observed that a recombinant construct with both eight amino acid residues (PFVNKQFN) deleted from the N-terminus and twenty-two amino acid residues (KNFTG LFEFYKLLCV RGIITSK) deleted from the C-terminus of the light chain of botulinum toxin A exhibits a reduced activity such that the effective concentration (EC_{50}) required to cleave the SNAP-25 substrate is nearly ten-fold greater than that of a similar construct with only the C-terminal twenty-two amino acid deletion (EC_{50} ΔN8ΔC22 =4663 pM *vs.* EC_{50}Δ C22 =566 pM). The recombinant light chain of botulinum toxin A was used as a control (EC_{50} rLC/A =7 pM), and, therefore, as compared to the rLC/A construct, a 666-fold greater concentration of the ΔNΔ8C22 construct is required. A recombinant light chain construct with the dileucine motif mutated to dialanine [rLC/A(LL-->AA)] also

exhibits reduced activity (EC_{50} rLC/A(LL-->AA) =184 pM); however, the effective concentration of the ΔN8ΔC22 construct is twenty-five fold greater than the rLC/A(LL-->AA) construct.

[00159] A modified light chain may include a light chain from botulinum toxins A, B, C1, D, E, F or G. One or multiple domains at the N- and/or C-terminus may be modified by addition, deletion or substitution. For example, a modified chimeric light chain component may include a light chain from BoNT/E modified by adding or replacing/substituting one or more N- and/or C-terminal end sequences derived from the BoNT/A light chain, thereby resulting in a chimeric BoNT/E-BoNT/A chimeric light chain with one or both terminal ends having one or more sequences which convey an increased or decreased ability to localize to a plasma membrane, increased or decreased biological persistence and/or an increased or decreased enzymatic activity.

[00160] A chimeric botulinum toxin can be constructed such that a C-terminal portion of the light chain of one botulinum toxin serotype replaces a similar C-terminal portion within the light chain of another botulinum toxin serotype. For example, the last twenty two amino acid residues bearing the dileucine motif from the C-terminus of the light chain of BoNT/A can replace the last twenty two amino acid residues of the C-terminus of the light chain of BoNT/E. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPKINSFNYNDPVNDRTILYIKPGGCQEFYKSFNIMKNIWIIPERNVIGTTPQDFHPPTSLKN
GDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGILLEELSKANPYLGNDNTPDNQFH
IGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSSNISLRNNYMPSNHGFGSIAIVTFSPE
YSFRFNDNSMNEFIQDPALTLMHELIHSLHGLYGAKGITTKYTITQKQNPLITNIRGTNIEEF
LTFGGTDLNIITSAQSNDIYTNLLADYKKIASKLSKVQVSNPLLNPYKDVFEAKYGLDKDA
SGIYSVNINKFNDIFKKLYSFTEFDLATKFQVKCRQTYIGQYKYFKLSNLLNDSIYNISEGYN
INNLKVNFRGQNANLNPRIITPITG**KNFTGLFEFYKLLCVRGIITSK**

(SEQ ID NO:124)

[00161] In the construct above, the majority of the amino acid sequence is derived from BoNT/E serotype, and the amino acids shown in bold underlined text

are derived from the last twenty two amino acid residues of the C-terminus of the light chain of BoNT/A which bears the dileucine motif.

[00162] In a further example, the first thirty amino acid residues from the N-terminus of the light chain of BoNT/A can replace the first thirty amino acid residues of the N-terminus of the light chain of BoNT/B. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMGRYYKAFKITDRIWIIPERYTFGYKPEDFN
KSSGIFNRDVCEYYDPDYLNTNDKKNIFFQTLIKLFNRIKSKPLGEKLLEMIINGIPYLGDRR
VPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNENETIDIGIQNHFASREGF
GGIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPALILMHELIHVLHGLYGIKVDDLP
IVPNEKKFFMQSTDTIQAEELYTFGGQDPSIISPSTDKSIYDKVLQNFRGIVDRLNKVLVCIS
DPNININIYKNKFKDKYKFVEDSEGKYSIDVESFNKLYKSLMLGFTEINIAENYKIKTRASYF
SDSLPPVKIKNLLDNEIYTIEEGFNISDKNMGKEYRGQNKAINKQAYEEISKEHLAVYKIQM
CKSVK (SEQ ID NO:125)

[00163] In the construct above, the majority of the amino acid sequence is derived from BoNT/B serotype, and the amino acids shown in bold underlined text are derived from the first thirty amino acid residues of the N-terminus of the light chain of BoNT/A.

[00164] Still further, the chimeric construct can have both N-terminal and the C-terminal replacements. For example, the first nine amino acid residues from the N-terminus of the light chain of BoNT/A can replace the first nine amino acid residues of the N-terminus of the light chain of BoNT/E. Additionally, in the same construct, the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/A can replace the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/E. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPFVNKQFNNDPVNDRTILYIKPGGCQEFYKSFNIMKNIWIIPERNVIGTTPQDFHPPTSLK
NGDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGILLEELSKANPYLGNDNTPDNQF
HIGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSSNISLRNNYMPSNHGFGSIAIVTFSP
EYSFRFNDNSMNEFIQDPALTLMHELIHSLHGLYGAKGITTKYTITQKQNPLITNIRGTNIEE
FLTFGGTDLNIITSAQSNDIYTNLLADYKKIASKLSKVQVSNPLLNPYKDVFEAKYGLDKD

ASGIYSVNINKFNDIFKKLYSFTEFDLATKFQVKCRQTYIGQYKYFKLSNLLNDSIYNISEGY
NINNLKVNFRGQNANLNPRIITPITG**KNFTGLFEFYKLLCVRGIITSK**

(SEQ ID NO:126)

[00165] In the construct above, the majority of the amino acid sequence is derived from BoNT/E serotype, and the amino acids shown in bold underlined text are derived from the first nine amino acid residues of the N-terminus and the last twenty-two amino acid residues of the C-terminus of the light chain of BoNT/A.

[00166] Similarly, the first nine amino acid residues from the N-terminus of the light chain of BoNT/A can replace the first nine amino acid residues of the N-terminus of the light chain of BoNT/B. Additionally, in the same construct, the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/A can replace the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/B. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPFVNKQFNYNDPIDNDNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKPEDFNK
SSGIFNRDVCEYYDPDYLNTNDKKNIFFQTLIKLFNRIKSKPLGEKLLEMIINGIPYLGDRRV
PLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNENETIDIGIQNHFASREGFG
GIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPALILMHELIHVLHGLYGIKVDDLPI
VPNEKKFFMQSTDTIQAEELYTFGGQDPSIISPSTDKSIYDKVLQNFRGIVDRLNKVLVCISD
PNININIYKNKFKDKYKFVEDSEGKYSIDVESFNKLYKSLMLGFTEINIAENYKIKTRASYFS
DSLPPVKIKNLLDNEIYTIEEGFNISDKNMGKEYRGQNKAINKQ**KNFTGLFEFYKLLCVR**
GIITSK (SEQ ID #127)

[00167] In the construct above, the majority of the amino acid sequence is derived from BoNT/B serotype, and the amino acids shown in bold underlined text are derived from the first nine amino acid residues of the N-terminus and the last twenty-two amino acid residues of the C-terminus of the light chain of BoNT/A.

[00168] Furthermore, the first nine amino acid residues from the N-terminus of the light chain of BoNT/A can replace the first nine amino acid residues of the N-terminus of the light chain of BoNT/F. Additionally, in the same construct, the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/A

can replace the last twenty-two amino acid residues from the C-terminus of the light chain of BoNT/F. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPFVNKQFNYNDPVNDDTILYMQIPYEEKSKKYYKAFEIMRNVWIIPERNTIGTNPSDFDP
PASLKNGSSAYYDPNYLTTDAEKDRYLKTTIKLFKRINSNPAGKVLLQEISYAKPYLGNDH
TPIDEFSPVTRTTSVNIKLSTNVESSMLLNLLVLGAGPDIFESCCYPVRKLIDPDVVYDPSNY
GFGSINIVTFSPEYEYTFNDISGGHNSSTESFIADPAISLAHELIHALHGLYGARGVTYEETIE
VKQAPLMIAEKPIRLEEFLTFGGQDLNIITSAMKEKIYNNLLANYEKIATRLSEVNSAPPEY
DINEYKDYFQWKYGLDKNADGSYTVNENKFNEIYKKLYSFTESDLANKFKVKCRNTYFIK
YEFLKVPNLLDDDIYTVSEGFNIGNLAVNNRGQSIKLNPKIIDKNFTGLFEFYKLLCVRGII
TSK (SEQ ID #128)

[00169] In the construct above, the majority of the amino acid sequence is derived from BoNT/F serotype, and the amino acids shown in bold underlined text are derived from the first nine amino acid residues of the N-terminus and the last twenty-two amino acid residues of the C-terminus of the light chain of BoNT/A.

[00170] In some embodiments, a light chain can be engineered such that one or more segments of the light chain of one or more toxin serotypes replace one or more segments of equal or unequal length within the light chain of another toxin serotype. In a non-limiting example of this kind of chimeric construct, fifty amino acid residues from the N-terminus of the light chain of BoNT/A can replace eight amino acid residues of the N-terminus of the light chain of BoNT/B, resulting in a net gain of fourty-two amino acids in length in the N-terminal region of the light chain chimera. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFYNDPIDN
DNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKPEDFNKSSGIFNRDVCEYYDPDY
LNTNDKKNIFFQTLIKLFNRIKSKPLGEKLLEMIINGIPYLGDRRVPLEEFNTNIASVTVNKLI
SNPGEVERKKGIFANLIIFGPGPVLNENETIDIGIQNHFASREGFGGIMQMKFCPEYVSVFNN
VQENKGASIFNRRGYFSDPALILMHELIHVLHGLYGIKVDDLPIVPNEKKFFMQSTDTIQAE
ELYTFGGQDPSIISPSTDKSIYDKVLQNFRGIVDRLNKVLVCISDPNININIYKNKFKDKYKF
VEDSEGKYSIDVESFNKLYKSLMLGFTEINIAENYKIKTRASYFSDSLPPVKIKNLLDNEIYTI
EEGFNISDKNMGKEYRGQNKAINKQAYEEISKEHLAVYKIQMCKSVK

(SEQ ID #129)

[00171] In the construct above, the majority of the amino acid sequence is derived from BoNT/B serotype, and the amino acids shown in bold underlined text are derived from the first fifty amino acid residues of the N-terminus of the light chain of BoNT/A.

[00172] In a non-limiting example of this kind of chimeric construct, the last fifty amino acid residues from the C-terminus of the light chain of BoNT/A can replace fifteen amino acid residues within the C-terminus of the light chain of BoNT/E, resulting in a net gain of thirty-five amino acids in the C-terminal region of the light chain chimera. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPKINSFNYNDPVNDRTILYIKPGGCQEFYKSFNIMKNIWIIPERNVIGTTPQDFHPPTSLKN
GDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGILLEELSKANPYLGNDNTPDNQFH
IGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSSNISLRNNYMPSNHGFGSIAIVTFSPE
YSFRFNDNSMNEFIQDPALTLMHELIHSLHGLYGAKGITTKYTITQKQNPLITNIRGTNIEEF
LTFGGTDLNIITSAQSNDIYTNLLADYKKIASKLSKVQVSNPLLNPYKDVFEAKYGLDKDA
SGIYSVNINKFNDIFKKLYSFTEFDLATKFQVKCRQTYIGQYKYFKLSNLLNDSIYNISEGYN
INNLKVNFRGQNANLNPRIITPGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFEF
YKLLCVRGIITSKNIVSVKGIRK

(SEQ ID #130)

[00173] In the construct above, the majority of the amino acid sequence is derived from BoNT/E serotype, and the amino acids shown in bold underlined text are derived from the last fifty amino acid residues of the C-terminus of the light chain of BoNT/A.

[00174] In a non-limiting example of this kind of chimeric construct, thirty amino acid residues from the N-terminus of the light chain of BoNT/A can replace ten amino acid residues of the N-terminus of the light chain of BoNT/E, resulting in a net gain of twenty amino acids in length in the N-terminal region of the chimera. Additionally, in the same construct, the last fifty amino acid residues from the C-terminus of the light chain of BoNT/A can replace the last fifty amino acid residues from the C-terminus of the light chain of BoNT/E. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPKINSFNYMPFVNKQFNYKDPVNGVDIAYIKIPNAGQMYIKPGGCQEFYKSFNIMKNI
WIIPERNVIGTTPQDFHPPTSLKNGDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGI
LLEELSKANPYLGNDNTPDNQFHIGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSSNI
SLRNNYMPSNHGFGSIAIVTFSPEYSFRFNDNSMNEFIQDPALTLMHELIHSLHGLYGAKGI
TTKYTITQKQNPLITNIRGTNIEEFLTFGGTDLNIITSAQSNDIYTNLLADYKKIASKLSKVQV
SNPLLNPYKDVFEAKYGLDKDASGIYSVNINKFNDIFKKLYSFTEFDLATKFQVKCRQTYI
GQYKYFKLSNLLNDSIYNISEGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFEFY
KLLCVRGIITSK (SEQ ID #131)

[00175] In the construct above, the majority of the amino acid sequence is derived from BoNT/E serotype, and the amino acids shown in bold underlined text are derived from the thirty amino acid residues of the N-terminus and the last fifty amino acid residues of the C-terminus of the light chain of BoNT/A.

[00176] In a non-limiting example of this kind of chimeric construct, thirty amino acid residues from the N-terminus of the light chain of BoNT/A can replace ten amino acid residues of the N-terminus of the light chain of BoNT/B, resulting in a net gain of twenty amino acids in length in the N-terminal region of the chimera. Additionally, in the same construct, the last fifty amino acid residues from the C-terminus of the light chain of BoNT/A can replace the last fifty amino acid residues from the C-terminus of the light chain of BoNT/B. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPVTINNFNMPFVNKQFNYKDPVNGVDIAYIKIPNAGQMIMMEPPFARGTGRYYKAFKI
TDRIWIIPERYTFGYKPEDFNKSSGIFNRDVCEYYDPDYLNTNDKKNIFFQTLIKLFNRIKSK
PLGEKLLEMIINGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPV
LNENETIDIGIQNHFASREGFGGIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPALIL
MHELIHVLHGLYGIKVDDLPIVPNEKKFFMQSTDTIQAEELYTFGGQDPSIISPSTDKSIYDK
VLQNFRGIVDRLNKVLVCISDPNININIYKNKFKDKYKFVEDSEGKYSIDVESFNKLYKSLM
LGFTEINIAENYKIKTRASYFSDSLPPVKIKNLLDNEIGFNLRNTNLAANFNGQNTEINNM
NFTKLKNFTGLFEFYKLLCVRGIITSK (SEQ ID #132)

[00177] In the construct above, the majority of the amino acid sequence is derived from BoNT/B serotype, and the amino acids shown in bold underlined text are derived from the thirty amino acid residues of the N-terminus and the last fifty amino acid residues of the C-terminus of the light chain of BoNT/A.

[00178] In a non-limiting example of this kind of chimeric construct, thirty amino acid residues from the N-terminus of the light chain of BoNT/A can replace ten amino acid residues of the N-terminus of the light chain of BoNT/F, resulting in a net gain of twenty amino acids in length in the N-terminal region of the chimera. Additionally, in the same construct, the last fifty amino acid residues from the C-terminus of the light chain of BoNT/A can replace the last fifty amino acid residues from the C-terminus of the light chain of BoNT/F. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPVAINSFN**MPFVNKQFNYKDPVNGVDIAYIKIPNAGQM**LYMQIPYEEKSKKYYKAFEI
MRNVWIIPERNTIGTNPSDFDPPASLKNGSSAYYDPNYLTTDAEKDRYLKTTIKLFKRINSN
PAGKVLLQEISYAKPYLGNDHTPIDEFSPVTRTTSVNIKLSTNVESSMLLNLLVLGAGPDIFE
SCCYPVRKLIDPDVVYDPSNYGFGSINIVTFSPEYEYTFNDISGGHNSSTESFIADPAISLAHE
LIHALHGLYGARGVTYEETIEVKQAPLMIAEKPIRLEEFLTFGGQDLNIITSAMKEKIYNNLL
ANYEKIATRLSEVNSAPPEYDINEYKDYFQWKYGLDKNADGSYTVNENKFNEIYKKLYSF
TESDLANKFKVKCRNTYFIKYEFLKVPNLLDDDIY**GFNLRNTNLAANFNGQNTEINNMN**
FTKLKNFTGLFEFYKLLCVRGIITSK (SEQ ID #133)

[00179] In the construct above, the majority of the amino acid sequence is derived from BoNT/F serotype, and the amino acids shown in bold underlined text are derived from the thirty amino acid residues of the N-terminus and the last fifty amino acid residues of the C-terminus of the light chain of BoNT/A.

[00180] In some embodiments, the swapped sequences can be derived from two different serotypes, resulting in a chimera with regions from three different serotypes in all. In this example, eight amino acid residues from the N-terminus of the light chain of BoNT/B can replace five amino acid residues of the N-terminus of the light chain of BoNT/E, resulting in a net gain of three amino acids in length in the N-terminal region of the chimera. Additionally, in the same construct, 30 amino acid residues including the dileucine repeat of the C-terminus of the light chain of BoNT/A can replace ten amino acid residues within the C-terminus of the light chain of BoNT/E, resulting in a net gain of 20 amino acids in the C-terminal region of the chimera. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPKINSFNYNDP***VTINNFNY***DRTILYIKPGGCQEFYKSFNIMKNIWIIPERNVIGTTPQDFHP
PTSLKNGDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGILLEELSKANPYLGNDNT
PDNQFHIGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSSNISLRNNYMPSNHGFGSIAI
VTFSPEYSFRFNDNSMNEFIQDPALTLMHELIHSLHGLYGAKGITTKYTITQKQNPLITNIRG
TNIEEFLTFGGTDLNIITSAQSNDIYTNLLADYKKIASKLSKVQVSNPLLNPYKDVFEAKYG
LDKDASGIYSVNINKFNDIFKKLYSFTEFDLATKFQVKCRQTYIGQYKYFKLSNLLNDSIYN
ISEGYNINNLKVNFRGQNANLNPRIITPITGRGLVKKIIRFCK<u>NNMNFTKLKNFTGLFEFY
KLLCVRGIITSK</u>

(SEQ ID #134)

[00181] In the construct above, the majority of the amino acid sequence is derived from BoNT/E serotype, and the amino acids shown in bold italicized text are derived from eight amino acid residues of the N-terminus of the light chain of BoNT/B and thirty amino acid residues shown in bold underlined text are derived from thirty amino acid residues of the C-terminus of the light chain of BoNT/A.

[00182] In a non-limiting example, eight amino acid residues from the N-terminus of the light chain of BoNT/B can replace five amino acid residues of the N-terminus of the light chain of BoNT/F, resulting in a net gain of three amino acids in length in the N-terminal region of the chimera. Additionally, in the same construct, 30 amino acid residues including the dileucine repeat of the C-terminus of the light chain of BoNT/A can replace ten amino acid residues within the C-terminus of the light chain of BoNT/F, resulting in a net gain of 20 amino acids in the C-terminal region of the chimera. The amino acid sequence of the entire light chain of such a chimeric construct is shown below:

MPVAINSFNYND***VTINNFNY***TILYMQIPYEEKSKKYYKAFEIMRNVWIIPERNTIGTNPSDF
DPPASLKNGSSAYYDPNYLTTDAEKDRYLKTTIKLFKRINSNPAGKVLLQEISYAKPYLGN
DHTPIDEFSPVTRTTSVNIKLSTNVESSMLLNLLVLGAGPDIFESCCYPVRKLIDPDVVYDPS
NYGFGSINIVTFSPEYEYTFNDISGGHNSSTESFIADPAISLAHELIHALHGLYGARGVTYEE
TIEVKQAPLMIAEKPIRLEEFLTFGGQDLNIITSAMKEKIYNNLLANYEKIATRLSEVNSAPP
EYDINEYKDYFQWKYGLDKNADGSYTVNENKFNEIYKKLYSFTESDLANKFKVKCRNTY
FIKYEFLKVPNLLDDDIYTVSEGFNIGNLAVNNRGQSIKLNPKIIDSIPDKGLVEK<u>NNMNFT
KLKNFTGLFEFYKLLCVRGIITSK</u>RK (SEQ ID #135)

[00183] In the construct above, the majority of the amino acid sequence is derived from BoNT/F serotype, and the amino acids shown in bold italicized text are

derived from eight amino acid residues of the N-terminus of the light chain of BoNT/B and thirty amino acid residues shown in bold underlined text are derived from thirty amino acid residues of the C-terminus of the light chain of BoNT/A.

EXAMPLE 14

[00184] The invention also provides for a light chain of a botulinum toxin B, C1, D, E, F or G comprising about the first 30 amino acids from the N-terminus of the light chain of botulinum toxin type A and about the last 50 amino acids from the C-terminus of the light chain of botulinum toxin type A. The first 30 amino acids of the N-terminus of type A here may be all or part, for example 2-16 contiguous or non contiguous amino acids, of the 30 amino acids. The last 50 amino acids here may be all or part, for example 5-43 contiguous or non-contiguous, amino acids of the 50 amino acids.

[00185] In some embodiments, such a light chain comprises about the first 20 amino acids from the N-terminus of the light chain of botulinum toxin type A and about the last 30 amino acids from the C-terminus of the light chain of botulinum toxin type A. The first 20 amino acids of the N-terminus of type A here may be all or part, for example 2-16 contiguous or non contiguous amino acids, of the 20 amino acids. The last 30 amino acids here may be all or part, for example 5-23 contiguous or non-contiguous, amino acids of the 30 amino acids.

[00186] In some embodiments, such a light chain comprises about the first 4 to 8, e.g. the first 8, amino acids from the N-terminus of the light chain of botulinum toxin type A and about the last 7 to 22, e.g. the last 22, amino acids from the C-terminus of the light chain of botulinum toxin type A. The first 8 amino acids of the N-terminus of type A here may be all or part, for example 2-7 contiguous or non contiguous amino acids, of the 7 amino acids. The last 22 amino acids here may be all or part, for example 5-16 contiguous or non-contiguous, amino acids of the 20 amino acids.

[00187] In some embodiments, the inclusion of about the first 30 amino acids from the N-terminus and about the last 50 amino acids from the C-terminus of the

light chain of type A replaces one or more amino acids at the N- and C-termini, respectively, of the light chain of botulinum toxin type B, C1, D, E, F or G. The first 30 amino acids of the N-terminus of type A here may be all or part, for example 2-16 contiguous or non contiguous amino acids, of the 30 amino acids. The last 50 amino acids here may be all or part, for example 5-43 contiguous or non-contiguous, amino acids of the 50 amino acids.

[00188] In some embodiments, the inclusion of about the 20 amino acids from the N-terminus and about the 30 amino acids from the C-terminus of the light chain of type A replaces one or more amino acids at the N- and C-termini, respectively, of the light chain of botulinum toxin type B, C1, D, E, F or G. The first 20 amino acids of the N-terminus of type A here may be all or part, for example 2-16 contiguous or non contiguous amino acids, of the 20 amino acids. The last 30 amino acids here may be all or part, for example 5-23 contiguous or non-contiguous, amino acids of the 30 amino acids.

[00189] In some embodiments, the inclusion of about the first 4 to 8, for example the first 8, amino acids from the N-terminus and about the last 7 to 22, for example the last 22, amino acids from the C-terminus of the light chain of type A replaces one or more amino acids at the N- and C-termini, respectively, of the light chain of botulinum toxin type B, C1, D, E, F or G. The first 8 amino acids of the N-terminus of type A here may be all or part, for example 2-7 contiguous or non contiguous amino acids, of the 7 amino acids. The last 22 amino acids here may be all or part, for example 5-16 contiguous or non-contiguous, amino acids of the 20 amino acids.

[00190] The invention also provides for a modified botulinum toxin comprising the light chain of described herein, including the ones described in the Examples above.

Example 15

[00191] **GENERATION OF LC/E CHIMERAS**

[00192] Truncation of 8 amino acids at the N-terminus of the LC/A completely disrupts plasma membrane localization. The LC/A(ΔN8) is cytoplasmic

with a distribution similar to LC/E. The sequences at the N-terminus of LC/A and LC/E are different (figure 1).

[00193] To generate the LC/E with the N-terminus of the LC/A we pursued two different approaches. The first one was to perform PCR on the native beluga LC/E gene with a 5' primer that contains the N-terminus of the LC/A. Primers for PCR were:

N-ter LC/A forward:

5'ACCGGATCCCCATTTGTTAATAAACAGTTTAATTATAATGA 3'

N-ter LC/A reverse:

5' CGCGAAGCTTCCTTATGCCTTTTACAGAAACAATATTTTTAC 3'

[00194] The PCR was performed with 0.4 µg of the plasmid template pQBI25fC3beluga LC/E, and 125ng of each primer. The cycling program was:

Denaturalizat: 95°C for 15 min

5 cycles:	94°C for 30 sec
	50°C for 30 sec
	72°C for 1 min
25 cycles:	94°C for 30 sec
	68°C for 30 sec
	72°C for 1 min

Extension: 72°C for 10 min

[00195] The first five cycles at low annealing temperature will allow the primer to anneal to the 5' sequence of the LC/E despite the differences in sequence. The second set of 25 cycles will use the product with the right sequence for further and more restricted amplification.

[00196] The second strategy to generate the LC/E chimera with the N-terminus of the LC/A was to mutate one amino acid as a time using site-directed mutagenesis. The primers designed were as follow:

1. Change the sequence on LC/E from Pro-Lys-Ile to Pro-Phe-Val:

   5' CCGGTTACCGGTACCGGATCCCCATTTGTTAATAGTTTTAATTAT 3'

2. Change the sequence Pro- Phe-Val-Asn-Ser to Pro-Phe-Val-Asn-Lys:

   5' CGGATCCCCATTTGTTAATAAATTTAATTATAATGATCCTGTT 3'

3. Insert a glutamine to complete the N-terminal sequence of the LC/A in the LC/E:

   5' GATCCCCATTTCTTAATAAACAGTTTAATTATAATGATCCTGTT 3'

[00197] Primers shown are on the sense strand, a complimentary primer corresponding to the antisense strand was also ordered and used in the PCR. The template used in the QuikChange mutagenesis was pQBI25fC3beluga LC/E.

[00198] We also analyzed the importance of the di-leucine motif present only in the LC/A by generating a motif in the LC/E in a similar area at the C-terminus (figure 2). The sequence in the LC/E reads LxxxII. Since Isoleucines can substitute for Leucines in some of the motif published we designed primers to generate ExxxII, and also primers to generate ExxxLL. Those mutations were done in the native LC/E gene and also in the LC/E containing the N-terminus of the LC/A.

[00199] We generated a total of 5 chimeras with all the combinations of the N-terminus of the LC/A and various stages of the di-leucine motif construction as seen in Figure 3. The complete DNA and annotated aminoacid sequence of the wild type LC/E, the chimeric LC/E with the N-terminus of the LC/A, the chimeric LC/E with the LC/A di-leucine motif at the C-terminus, and the full chimeric LC/E with both the N-terminus and the di-leucine motif from LC/A are shown in Figures 14-17. We have tested all those mutants for expression, activity, and subcellular localization.

[00200] **Cell Lines and Growth Conditions**

[00201] **SH-SY5Y** (Human Neuroblastoma cell line) cells were cultured in Costar brand polystyrene flasks with vented caps. Growth media consisted of Minimum Essential Medium with Earle's salts and L-glutamine, F-12 Nutrient Mixture (Ham) with L-glutamine, 10% Fetal Bovine Serum (heat-inactivated), Non-Essential Amino-Acids, HEPES, L-Glutamine, Penicillin / Streptomycin. PC-12 (Rat Chromaffin Pheochromocytoma cell line) cells were maintained on Collagen IV

coated BD Biocoat dishes (BD Biosciences, Bedford, MA). Growth media consisted of RPMI-1640 with L-glutamine, 10% Horse Serum (heat-inactivated), 5% Fetal Bovine Serum (heat-inactivated), HEPES, D-Glucose (Sigma), Sodium Pyruvate, Penicillin / Streptomycin. All (except D-Glucose, Sigma) growth media components were Gibco products purchased from Invitrogen and all cell lines were cultured and maintained at 37°C with 7.5% CO_2.

[00202] Transient transfection:

[00203] The day before transfection, SH-SY5Y cells were plated at 1 x 10^6 into 6-well plates. Transfections were carried out by diluting LipofectAmine 2000 (Invitrogen, Carlsbad, CA) at 60μl per ml in OPTI-MEM Reduced Serum Medium following incubation at RT for 5 min. Next, we diluted 20 μg DNA per ml in OPTI-MEM, then combined equal amount of DNA mix and LipofectAmine 2000 (LF) mix, and incubated at RT for another 20 min. Meanwhile, the culture medium in the plates were replaced with 2 ml of serum free medium, 0.5 ml of DNA + LF mix was then added into the wells and incubated at 37°C CO_2 incubator for 6 hours. After the incubation the medium was removed and replaced with 10% FCS culture medium. The cells were harvested 24 hours post transfection for further analysis. For confocal analysis, transfections were usually performed in 2-well culture slices with proportionally reduced amount of reagents.

[00204] Transfection into PC12 cells were performed as follow: PC12 cells were plated the day before transfection in Collagen IV coated dishes at 10 x 10^6 cells per 100 mm dishes and 2 x 10^6 cells per 60 mm dishes. Plates were transfected with 20μg or 10μg respectively with Lipofectamine 2000 in OPTI-MEM media. 48 hours after transfection, cells were placed on differentiation media and collected for western blots or fixed in 4% paraformaldehyde for confocal imaging.

[00205] Western Blot Analysis:

[00206] Cells were collected in 15 ml Falcon tubes, washed once with 1ml of PBS, and then transferred to 1.5 ml microcentrifuge tubes. Cells were lysed in 0.5 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol and 1% triton X-100) on rotator at 4°C for 1 hour. Lysed cells were spun

down at 5000 rpm at 4°C for 10 min to eliminate debris; supernatants were transferred to fresh siliconized tubes. Protein concentrations were measured by the Bradford's method and resuspended in 1 x SDS sample buffer at 1mg/ml or higher concentration. Samples were boiled for 5 min, 20 to 40 µl of the samples were loaded on 4-12% Tris-HCl gels. Proteins were transferred to PVDF membranes, and blocked in 5% non-fat milk in TBST buffer for 1 hour at room temperature. The cleaved SNAP25 was detected with antiSNAP25$_{197}$ antibody or with antiSNAP25$_{180}$ diluted in blocking buffer; blot was washed extensively, and the bound antibody was detected with horseradish peroxidase conjugated to species-specific antibody.

[00207] When we needed to detect SNAP25$_{206}$, SNAP25$_{197}$ and SNAP25$_{206}$ in the same blot using the Monoclonal antibody SMI-81 to the N-terminus of the SNAP25, cell lysates were run on a 12% Bis-Tris gel to allow separation of the cleaved SNAP25.

[00208] We used the Typhoon 9410 Imager (Amersham) for Western Blot Analysis instead of traditional film. After the final washes the membrane was reacted with ECL Plus western blot detection reagent (Amersham) rather than SuperSignal reagent used previously, blot was incubated at room temperature for 5 min to develop. The choice of pixel size and PMT voltage settings will depend on the individual blot. Membranes were scanned and quantified using Typhoon Scanner and Imager Analysis software.

[00209] SH-SY5Y Transient Transfections for SNAP-25 Immunocytochemistry

[00210] One day before transfection, SH-SY5Y cells were plated in 60 mm tissue culture dishes at densities of 1.5 x10^6 or 1.6 x10^6 cells per dish to achieve 90-95% confluence at the time of transfection. Transfections were performed by diluting 25 µl of Lipofectamine™ 2000 (Invitrogen) in 0.5 ml Opti-MEM® I Reduced Serum Medium (Invitrogen) followed by incubation at room temperature for 5 min. DNA (10 µg) was diluted in 0.5 ml Opti-MEM® I Reduced Serum Medium. The diluted DNA was mixed gently with the diluted Lipofectamine™ 2000. This mixture was incubated for 20 min at room temperature. Meanwhile, the culture medium in the

plates was replaced with 2 ml of serum-free and antibiotic-free medium. The DNA plus Lipofectamine™ 2000 complex was added drop-wise to the cells and mixed into the culture medium by rocking the plates back and forth. Cells were incubated at 37°C, 7.5% CO_2 for 24 hours. Transfection efficiencies were determined by viewing cells with the fluorescence microscope. Transfection efficiencies obtained with the GFP construct were ca. 40-50%. Lower transfection efficiencies were observed with the GFP-LCE constructs, ca. 10-15%. Antibiotic selection of cells was accomplished in complete medium containing 0.5 mg/ml Geneticin G418 (Invitrogen) for 48 hours before proceeding with the immunocytochemistry.

[00211] PC-12 Transient Transfections for SNAP-25 Immunocytochemistry

[00212] Cells were plated one day before transfection in Collagen IV coated dishes (BD Biosciences) at 1-2 x 10^6 cells per 60 mm dish. Plates were transfected using 10 µg DNA and 25 µl Lipofectamine™ 2000 (each diluted in 0.5 ml Opti-MEM® I Reduced Serum Medium). The cells were incubated with the DNA / Lipofectamine™ 2000 complex for 24 hours in serum- and antibiotic-free medium at 37°C, 7.5% CO_2. The transfection medium was replaced with complete growth medium (serum and antibiotics included) containing 0.5 mg/ml G418 (antibiotic selection) and incubation continued at 37°C, 7.5% CO_2 for a further 48 hours. Cells were placed in differentiation medium (RPMI-1640 with L-glutamine, D-Glucose (Sigma), Sodium Pyruvate, Penicillin / Streptomycin, BSA (ALBUMAX II, lipid rich), N2-Supplement) containing Nerve Growth Factor (NGF) (Harlan Bioproducts for Science, Indianapolis, IN) at 50 ng/ml final concentration for 24 hours and stained with antibodies specific for GFP, $SNAP25_{206}$ and $SNAP25_{180}$ (Table 6).

Table 6: List of antibodies used in the immunocytochemistry experiments

Antibody	Source	Dilution Used	Specificity / Immunogen
1A3A7, ascites IgG1-K, mouse monoclonal	Allergan	1:100, 1:50	Anti-$SNAP25_{180}$ cleavage product. Does not cross-react

			with $SNAP25_{206}$
1G8C11, ascites IgG1-K, mouse monoclonal	Allergan	1:100, 1:50	Anti-$SNAP25_{180}$ cleavage product. Does not cross-react with $SNAP25_{206}$
1C9F3, ascites IgG1-K, mouse monoclonal	Allergan	1:100, 1:50	Anti-$SNAP25_{180}$ cleavage product. Does not cross-react with $SNAP25_{206}$
Anti-GFP(FL), rabbitpolyclonal IgG	Santa Cruz Biotechnologies	1:100	Full length GFP (amino acids 1-238 of *Aequorea Victoria* origin)
Anti-GFP, mouse monoclonal IgG2	Abcam Inc.	1:100	Full length GFP (amino acids 1-246 of *Aequorea Victoria* origin)
Anti-SNAP-25, rabbit polyclonal	Stressgen Biotechnologies	1:100	Amino acids 195-206 of mouse / human / chicken SNAP-25. Specific for $SNAP25_{206}$
Alexa Fluor 568 Goat Anti-Rabbit IgG (H+L), highly cross-absorbed	Molecular Probes	1:100	Secondary for $SNAP\text{-}25_{206}$ detection
Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L)	Molecular Probes	1:100, 1:200	Secondary for rabbit polyclonal to GFP(FL)
Alexa Fluor 568 Goat Anti-Mouse IgG (H+L)	Molecular Probes	1:100	Secondary for mouse monoclonals to $SNAP25_{180}$

Alexa Fluor 488 Goat Anti-Mouse IgG (H+L)	Molecular Probes	1:100	Secondary for mouse monoclonal to GFP

[00213] Immunocytochemistry using Fixative Paraformaldehyde for $SNAP25_{206}$ and $SNAP25_{180}$

[00214] Growth medium was removed from cells by aspiration and cells were washed twice with PBS (Invitrogen, Carlsbad, CA). Cells were fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences, Washington, PA) for 15 to 30 min at room temperature and washed in three changes of PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and washed in PBS a total of three times. Cells were again permeabilized in ice-cold methanol for 6 min at -20°C. Methanol was removed by aspiration and dishes inverted to allow the cells to dry at room temperature. Wells were drawn around cells using a Pap pen (Zymed, San Francisco, CA) and cells were washed and rehydrated in six changes of PBS. Cells were blocked with 100 mM glycine in PBS for 30 min at room temperature followed by three washes in PBS. Cells were incubated in 0.5% BSA in PBS for 30 min at room temperature washed in three changes of PBS before addition of the primary antibodies diluted in 0.5% BSA in PBS (Table 6). Cells were incubated at room temperature in a humid chamber for 2 hours or at 4°C overnight. Primary antibody was removed by a PBS wash without incubation and was followed by three 5 min washes in PBS. Cells were incubated with the fluorescently labeled secondary antibodies (Alexa Fluor Anti-Mouse or Anti-Rabbit Antibodies, Molecular Probes, Table 6) diluted in 0.5% BSA/PBS for 1 hour at room temperature in a humid chamber and washed in PBS. Cells were mounted using Vectashield® Mounting Medium (Vector, Burlingham, CA) and coverslipped. Cells were stored at 4°C before viewing with a Leica confocal microscope

[00215] RESULTS

[00216] Generation of LC/E chimeras containing the localization signals for LC/A

[00217] We have identified sequences in the N-terminus and C-terminus of the LC/A that are important for localization of the protein to the plasma membrane. Deletion of the first 8 amino acids at the N-terminus causes complete loss of plasma membrane localization, with the LC/A(ΔN8) localizing in the cytoplasm with nuclear exclusion. Disruption of the di-leucine motif at the C-terminus produces also changes in localization. To validate those signals we generated chimeras between the LC/A and the LC/E, since both cleave the same substrate at different sites, but have different subcellular localization and duration of action. LC/E localizes to the cytoplasm and lasts 1-2 weeks. We generated the following constructs using the native beluga LC/E sequence: LC/E(N-LCA), LC/E(ExxxII), LC/E(ExxxLL), LC/E(N-LCA/ExxxII), and LC/E(N-LCA/ExxxLL) (Figure 3 and Table 7) in order to analyze the effect of each signal by itself, and the effect of both signals combined. These constructs were transfected into the human neuroblastoma cell line SH-SY5Y. Cell lysates were prepared and the activity of the mutants was analyzed with the SMI-81 antibody that recognizes both cleaved and intact SNAP25 (Figure 4).

[00218] **Table 7:** Explanation of the chimeric LC/E's generated and transfected into PC-12 and SH-SY5Y cells.

Name	Contains GFP attached to:
GFP-LCE	Wild-type-LC/E
GFP-LCE Nterm LCA	LC/E with the first eight amino-acid of LC/A at the N-terminus
GFP-LCE (ExxxII)	LC/E with a di-isoleucine motif in C-terminal region (di-isoleucine can substitute for leucine in the motif)
GFP-LCE (ExxxLL)	LC/E with a di-leucine motif in C-terminal region
GFP-LCE Nterm LCA	LC/E with the first eight amino-acid of LC/A at the N-

(ExxxII)	terminus and di-isoleucine motif in C-terminal region
GFP-LCE Nterm LCA (ExxxLL)	LC/E with the first eight amino-acid of LC/A at the N-terminus and dileucine motif in C-terminal region

[00219] All the chimeras expressed in SH-SY5Y are able to cleave $SNAP25_{206}$ into $SNAP25_{180}$, and they do not cleave the substrate at any other sites (figure 4). The preliminary data from the two experiments performed shows different levels of activity of the chimeras when compared to native LC/E. We could not distinguish if those differences are due to lower or higher levels of expression of the mutants, or to true changes on catalytic activity, because there was not enough material to run an immunoprecipitation to detect the chimeric LC/Es. Figure 5 shows the results of a new set of three independent experiments transfecting the chimeric LC/Es into PC12 and SH-SY5Y cells. The levels of expression of each construct are different but all of them retain catalytic activity towards the cleavage of SNAP25. For some of the constructs the catalytic activity seemed diminished when compared with the wild-type LC/E but those changes can only be confirmed by expressing the protein recombinantly and performing ELISA or GFP assays.

[00220] Work previously published by Fernandez-Salas, Steward et al. (PNAS 101, 3208-3213, 2004) showed the sub-cellular localization of GFP-LC/A and GFP-LC/E proteins in differentiated PC-12 cells (Figure 6). GFP-LC/A localized in a punctate manner in specific areas in the plasma membrane of the cell body and the neurites, with no localization of GFP-LC/A protein in the cytoplasm of the cells (Figure 6A.). GFP-LC/E showed cytoplasmic localization with nuclear exclusion. Cells displayed rounded morphology and lack of neurites even in differentiation medium (Figure 6B.).

[00221] The results in Figure 6 demonstrate that the light chains from BoNT serotypes A and E are directed to distinct sub-cellular compartments. To identify sequences of importance in directing LC/A localization to the plasma membrane, PC-12 and SH-SY5Y cells were transiently transfected with GFP-LC/E and GFP-

LC/E (N-LCA/ExxxLL) plasmid constructs. Transfected SH-SY5Y cells were selected in growth medium containing 0.5 mg/ml G418 for 2 days before staining with the Anti-SNAP25 and Anti-GFP antibodies. The transfected PC-12 cells were exposed to selection medium for 2 days followed by differentiation medium (containing NGF at 50 ng/ml final concentration) for 24 hours before staining.

[00222] Staining for the GFP portion of the LC/E chimera demonstrated that most of the GFP-LCE(N-LCA/ExxxLL) protein was localized at the plasma membrane in a punctuate manner (figure 7A and C), similar to the previously reported GFP-LC/A localization (Fig. 6A).The mouse monoclonal antibodies to $SNAP25_{180}$ used in this staining have high background staining but the cells expressing LC/E displayed a stronger signal. Moreover, cells expressing the GFP-LCE (N-LCA/ExxxLL) chimera contain $SNAP25_{180}$ that remains in the cytoplasm.

[00223] GFP staining shown in Figures 8A and 8C demonstrates the punctate localization of GFP-LC/E (N-LCA/ExxxLL) protein at the plasma membrane of the cells, similar to the GFP-LC/A localization presented in Figure 6A. The cells that are expressing the GFP-LC/E (N-LCA/ExxxLL) chimera protein (indicated by arrows corresponding to cells 'a', 'b' and 'e' in Fig. 8A and 8C) do not show staining with Anti-$SNAP25_{206}$ antibody (cells 'a', 'b' and 'e' in Figure 8B and 8D). This confirms the proteolytic activity of the GFP-LC/E (N-LCA/ExxxLL) protein expressed in these cells, as demonstrated by its ability to cleave $SNAP25_{206}$. On the contrary, cells depicted 'c' and 'd' in Figure 8C that are not expressing the GFP-LC/E (N-LCA/ExxxLL) protein give a good signal for the presence of full length $SNAP25_{206}$ protein as indicated by cells 'c' and 'd' in Figure 8D.

[00224] The GFP-LC/E protein is shown to localize to a structure within the cytoplasm in a punctate manner as previously reported in Fernandez-Salas, Steward et al. and Fernandez-Salas, Ho et al. (Movement Disorders 19, S23-S34., 2004) and shown in Figure 6B. $SNAP25_{180}$ protein also localizes in the cytoplasm of cells expressing GFP-LCE and appears to be in a granular structure (Figure 9B).

[00225] **SH-SY5Y Localization of GFP-LC/E**

[00226] GFP-LC/E protein localizes in the cytoplasm of SH-SY5Y cells. Notice the large area of GFP exclusion in the nuclei of both cells shown in Figure 10.

[00227] The GFP-LCE (N-LCA/ExxxLL) chimera was also expressed in SH-SY5Y cells. Staining with an anti-GFP antibody (Figure 11) demonstrated localization at the plasma membrane, similar to that seen for the chimera expressed in PC-12 cells.

[00228] The plasmid encoding chimeric GFP-LC/E (N-LCA/ExxxLL) was transfected into the human neuroblastoma cell line SH-SY5Y. The chimeric protein was expressed and dishes were fixed for immunostaining. Cells stained positive for GFP (monoclonal antibody) (indicated by arrows in Fig. 12A and 12C) show the GFP-LCE (N-LCA/ExxxLL) protein localized to the plasma membrane. Full length $SNAP25_{206}$ protein is not detected in cells expressing the GFP-LCE (N-LCA/ExxxLL) protein as indicated by the arrows in Figure 12B and 12D. This suggests the GFP-LCE (N-LCA/ExxxLL) chimera protein is functional and cleaves $SNAP25_{206}$.

[00229] To further confirm activity the transfected cells were also stained with the GFP and the monoclonal antibodies to $SNAP25_{180}$. Staining for GFP (polyclonal antibody) showed the GFP-LC/E (N-LCA/ExxxLL) protein localized at the plasma membrane as shown in the previous figure (Fig. 13A). The 1A3A7 ascites mouse monoclonal antibody used to stain $SNAP25_{180}$ has high background staining and is very weak (arrows in Fig. 13B indicate groups of cells positive for $SNAP25_{180}$ staining. This antibody was used at 1:100 dilution in this early experiment. However, cells expressing the GFP-LCE (N-LCA/ExxxLL) protein in Figure 13A contain $SNAP25_{180}$ in the cytoplasm as indicated by arrows in Figure 13B.

[00230] The GFP-LC/E fusion protein has previously been demonstrated to localize in the cytoplasm of PC12, HIT-T15, and HeLa cells. Adding the N-terminal LC/A signal (8 amino acids) and the C-terminal di-leucine motif of LC/A (ExxxLL) into the LCE protein sequence (GFP-LCE (N-LCA/ExxxLL) dramatically changed the sub-cellular localization of LC/E. Adding the LC/A localization signals to LC/E directed localization of the LC/E (N-LCA/ExxxLL) chimera to the plasma

membrane, validating these motifs/sequences as important signals for LC/A localization. Cleavage of SNAP25 by LC/E was detected using antibodies to the N-terminus of SNAP25. Co-staining with antibodies to full length $SNAP25_{206}$ demonstrated a loss of intact SNAP25 in cells expressing the functional GFP-LCE (N-LCA/ExxxLL) chimera protein. $SNAP25_{180}$ protein was also detected in the cytoplasm of cells expressing GFP-LCE (N-LCA/ExxxLL), indicating LC/E SNAP25 proteolysis. Further work will need to be done to optimize staining of $SNAP25_{180}$ cleavage product in the paraformaldehyde fixed cells because the anti-$SNAP25_{180}$ antibodies used in this study were weak. To identify compartments where the LC/A, LC/E and LC/E (N-LCA/ExxxLL) proteins reside, a panel of dyes and antibodies specific for cytoplasmic organelles and plasma membrane proteins (channels and receptors) will be employed. PC-12 cells have been transfected with wild-type GFP-LC/E, GFP-LC/E (ExxxLL), GFP-LC/E (ExxxII), GFP-LC/E (N-LCA), GFP-LC/E (N-LCA/ExxxII) and wild-type GFP-LC/A constructs. Localization of these chimera proteins will confirm sequences of importance in LC/A localization. The LC/E chimera containing the N-terminus of the LC/A and the di-leucine motif presents a very distinct localization and may constitute a LC/E with a longer duration of action.

[00231] While this invention has been described with respect to various specific examples and embodiments, it is to be understood that the invention is not limited thereto and that it can be variously practiced with the scope of the following claims. All articles, references, publications, and patents set forth above are incorporated herein by reference in their entireties.

What is claimed is:

1. A modified toxin comprising a modified light chain of a botulinum toxin type E, wherein the modified light chain comprises:

an amino acid sequence SEQ ID NO: 144 (PFVNKQFN) at the N-terminal of the modified light chain; and

an amino acid sequence SEQ ID NO: 112 (xExxxLL) at the C-terminal of the modified light chain, wherein x is any amino acid.

2. The toxin of claim 1 wherein the amino acid sequence SEQ ID NO: 144 (PFVNKQFN) is part of the first 12 amino acids of the N-terminal of the modified light chain, and the amino acid sequence SEQ ID NO: 112 (xExxxLL) is part of the last 27 amino acids of the C-terminal of the modified light chain.

3. The toxin of claim 1 wherein the amino acid sequence SEQ ID NO: 144 (PFVNKQFN) is part of the first 8 amino acids of the N-terminal of the modified light chain, and the amino acid sequence xExxxLL (SEQ ID NO: 112) is part of the last 23 amino acids of the C-terminal of the modified light chain.

4. The toxin of claim 1 wherein:

the amino acid sequence SEQ ID NO: 144 (PFVNKQFN) is the first 8 amino acids of the N-terminal of the modified light chain; and

the amino acid sequence SEQ ID NO: 112 (xExxxLL) is prior to the last 16 amino acids at the C-terminal of the modified light chain.

5. The toxin of claims 1, 2, 3, or 4 wherein the toxin further comprises a heavy chain of a Clostridial toxin.

6. The toxin of claim 5 wherein the heavy chain is a heavy chain of a botulinum toxin type A, B, C_1, D, E, F, or G.

7. The toxin of claim 5 wherein the heavy chain is a heavy chain of a botulinum toxin type E.

8. A modified toxin comprising an amino acid sequence of SEQ ID NO: 142.

9. The toxin of claim 8 further comprising a heavy chain of a Clostridial toxin.

10. The toxin of claim 9 wherein the heavy chain is a heavy chain of a botulinum toxin type A, B, C_1, D, E, F, or G.

11. The toxin of claim 9 wherein the heavy chain is a heavy chain of a botulinum toxin type E.

12. A nucleic acid sequence comprising a region that encodes an amino acid sequence SEQ ID NO: 142.

13. The nucleic acid sequence of claim 12 comprising SEQ ID NO: 143.

14. The nucleic acid sequence of claim 12 further comprising a region that encodes a heavy chain of a Clostridial toxin.

15. The nucleic acid sequence of claim 14 wherein the heavy chain is a heavy chain of a botulinum toxin type A, B, C_1, D, E, F, or G.

16. The nucleic acid sequence of claim 14 wherein the heavy chain is a heavy chain of a botulinum toxin type E.

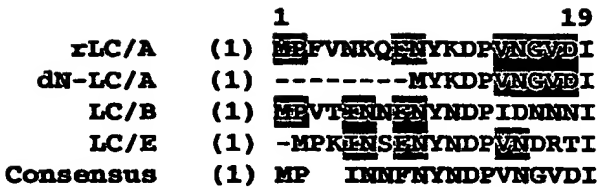

1 19
rLC/A (1) MPFVNKQFNYKDPVNGVDI
dN-LC/A (1) --------MYKDPVNGVDI
LC/B (1) MPVTINNFNYNDPIDNNNI
LC/E (1) -MPKINSFNYNDPVNDRTI
Consensus (1) MP INNFNYNDPVNGVDI

Figure 1

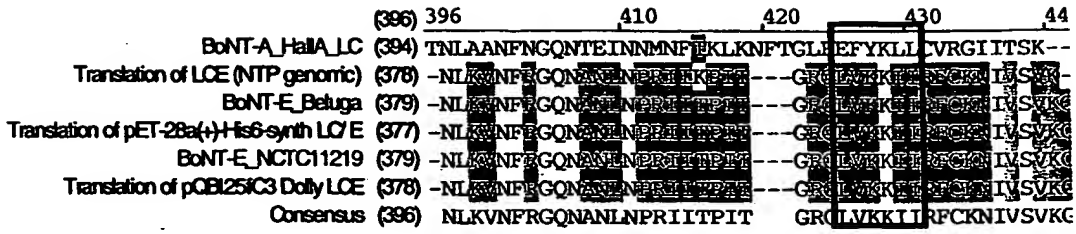

(396) 396 410 420 430 44
BoNT-A_HallA_LC (394) TNLAANFNGQNTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSK--
Translation of LCE (NTP genomic) (378) -NLKVNFRGQNANLNPRIITPIT---GRGLVKKLIRFCKNIVSVK-
BoNT-E_Beluga (379) -NLKVNFRGQNANLNPRIITPIT---GRGLVKKLIRFCKNIVSVK
Translation of pET-28a(+)-His6-synth LC/E (377) -NLKVNFRGQNANLNPRIITPIT---GRGLVKKLIRFCKNIVSVK
BoNT-E_NCTC11219 (379) -NLKVNFRGQNANLNPRIITPIT---GRGLVKKLIRFCKNIVSVK
Translation of pQBI25fC3 Dolly LCE (378) -NLKVNFRGQNANLNPRIITPIT---GRGLVKKLIRFCKNIVSVK
Consensus (396) NLKVNFRGQNANLNPRIITPIT GRGLVKKLIRFCKNIVSVK

Figure 2

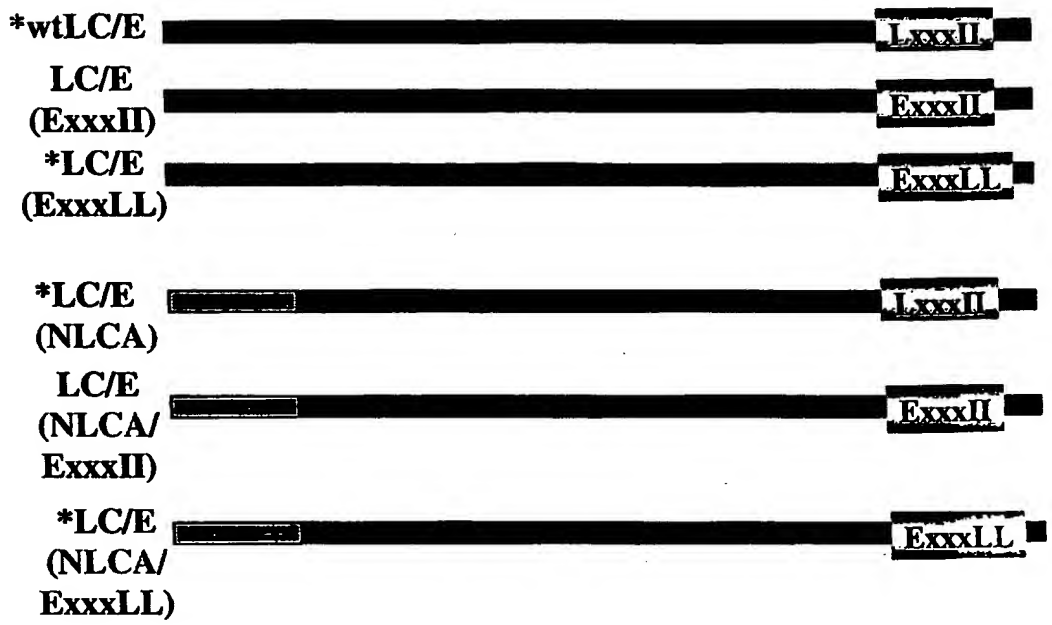

*wtLC/E
LxxxII
LC/E
(ExxxII)
ExxxII
*LC/E
(ExxxLL)
ExxxLL
*LC/E
(NLCA)
LxxxII
LC/E
(NLCA/
ExxxII)
ExxxII
*LC/E
(NLCA/
ExxxLL)
ExxxLL

Figure 3

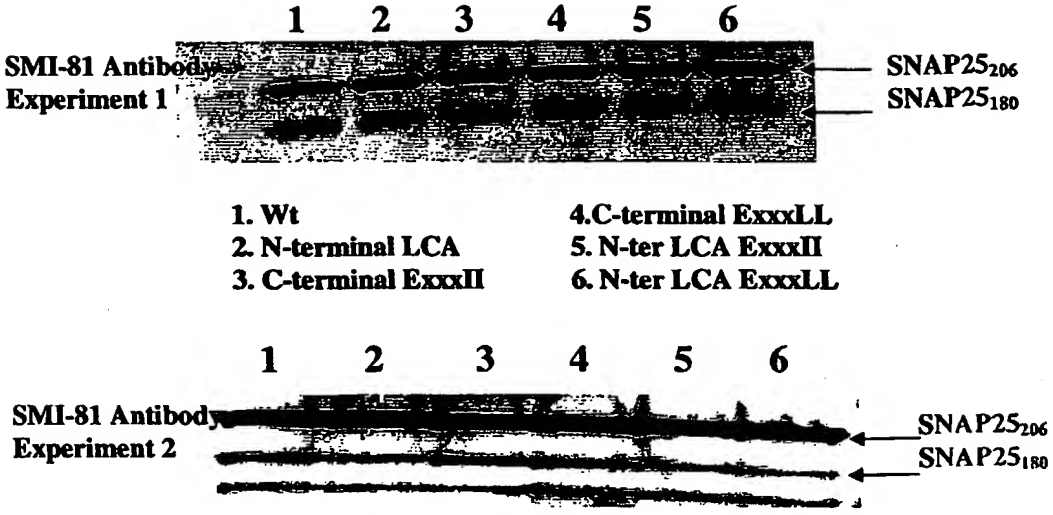
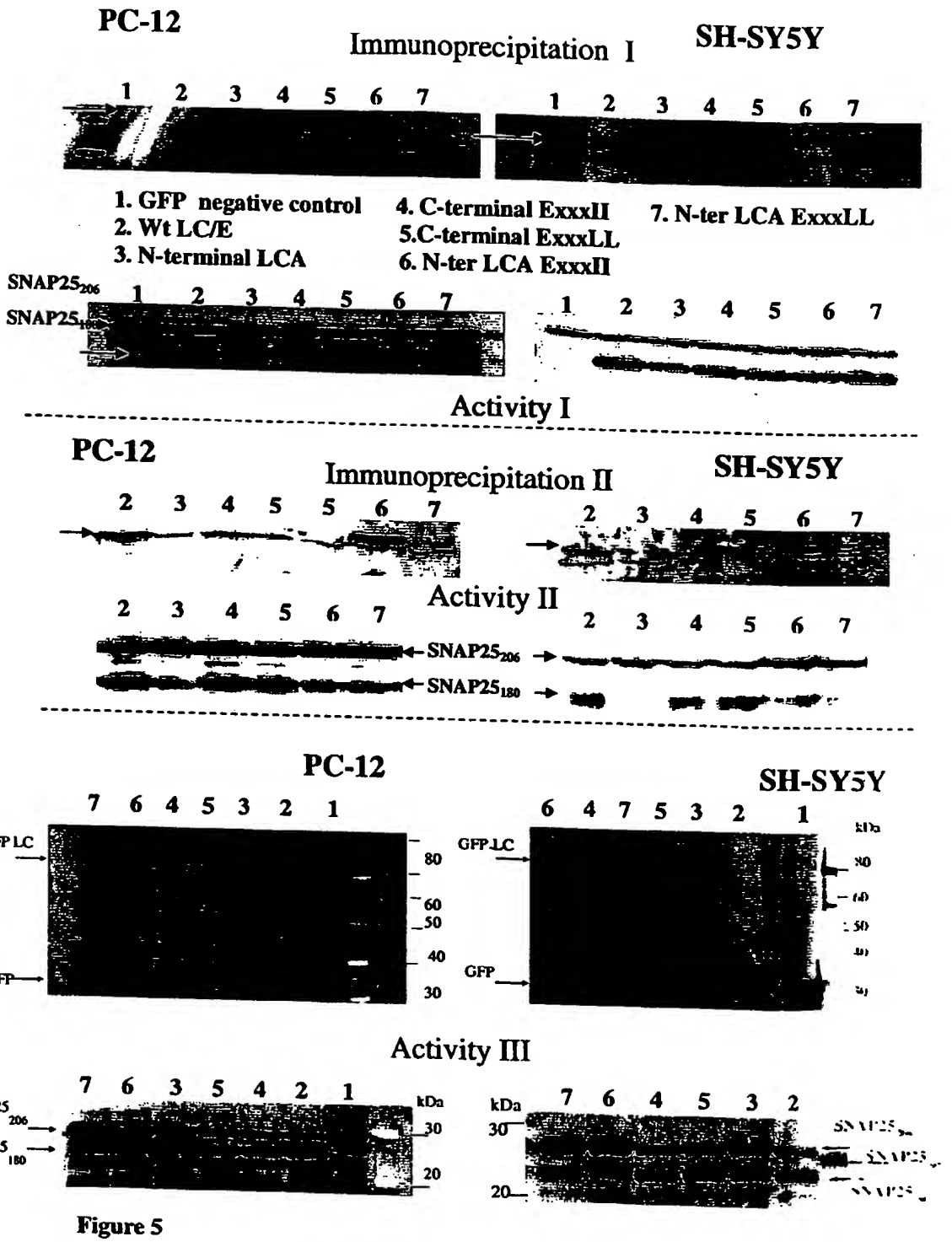

1 2 3 4 5 6
SMI-81 Antibody
Experiment 1
SNAP25206
SNAP25180
1. Wt
2. N-terminal LCA
3. C-terminal ExxxII
4.C-terminal ExxxLL
5. N-ter LCA ExxxII
6. N-ter LCA ExxxLL
1 2 3 4 5 6
SMI-81 Antibody
Experiment 2
SNAP25206
SNAP25180

Figure 4


PC-12
Immunoprecipitation I
SH-SY5Y
1 2 3 4 5 6 7
1. GFP negative control
2. Wt LC/E
3. N-terminal LCA
4. C-terminal ExxxII
5.C-terminal ExxxLL
6. N-ter LCA ExxxII
7. N-ter LCA ExxxLL
SNAP25206
SNAP25180
Activity I
PC-12
Immunoprecipitation II
SH-SY5Y
Activity II
Activity III
GFP LC
GFP
kDa
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50
40
30
20
SNAP25 206
SNAP25 180

Figure 5

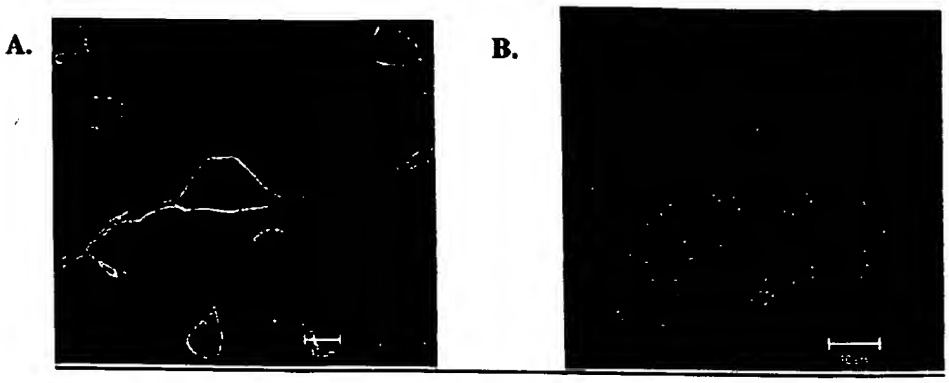

Figure 6

PC-12 Localization of GFP-LC/E (NLCA/ExxxLL)

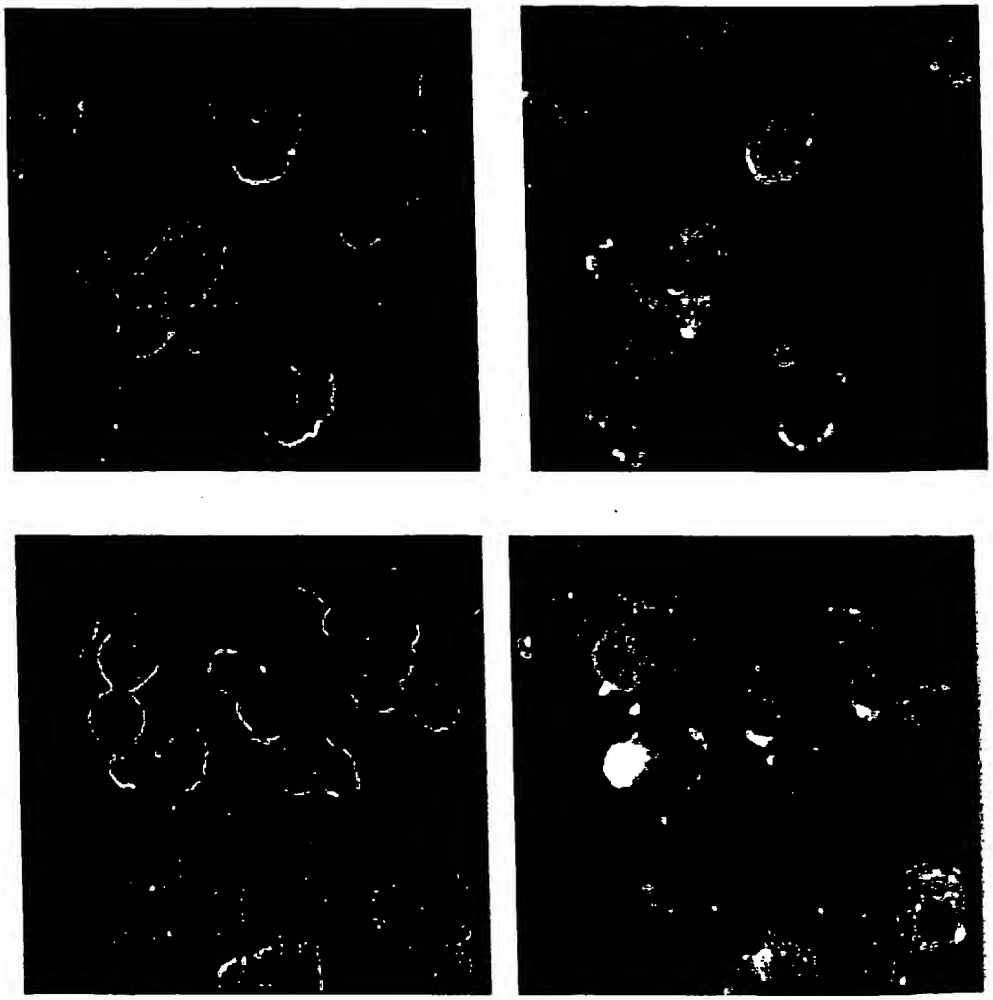

Figure 7

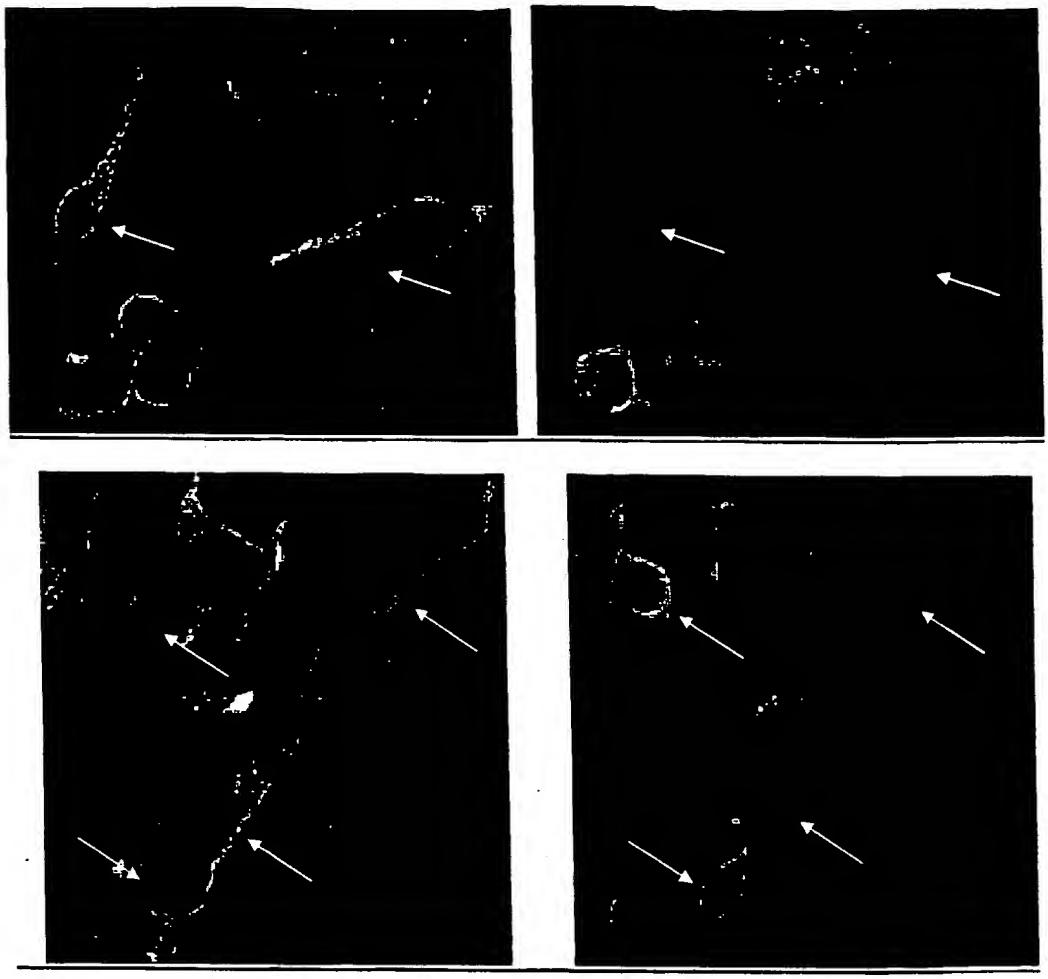

Figure 8

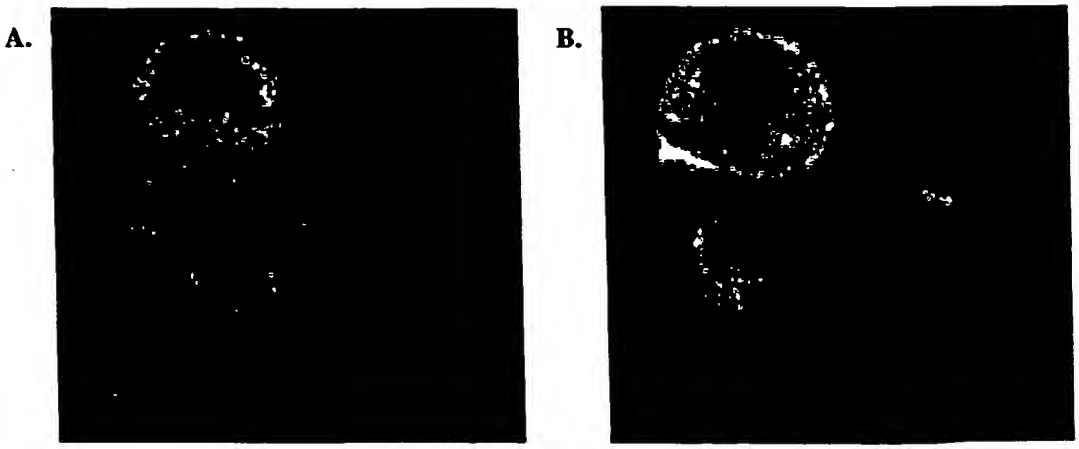

Figure 9

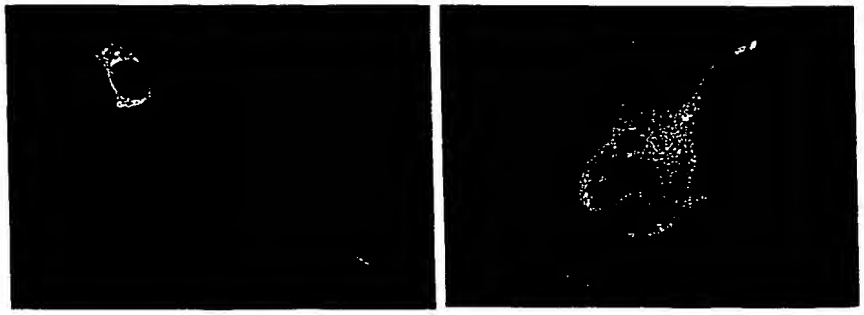

Figure 10

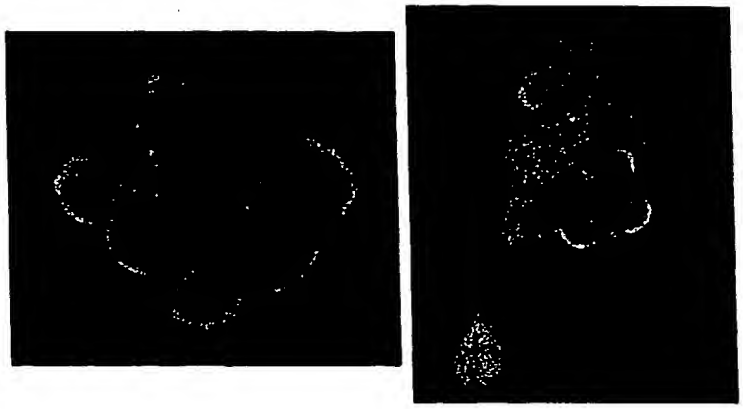

Figure 11

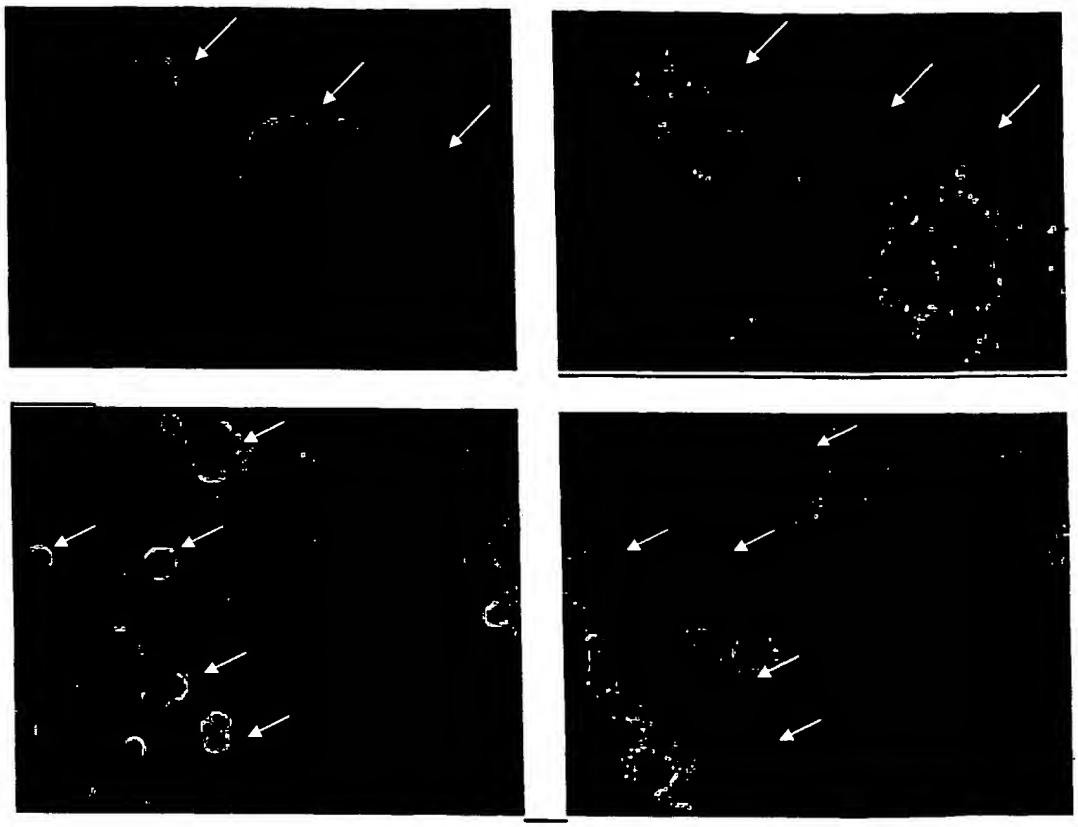

Figure 12

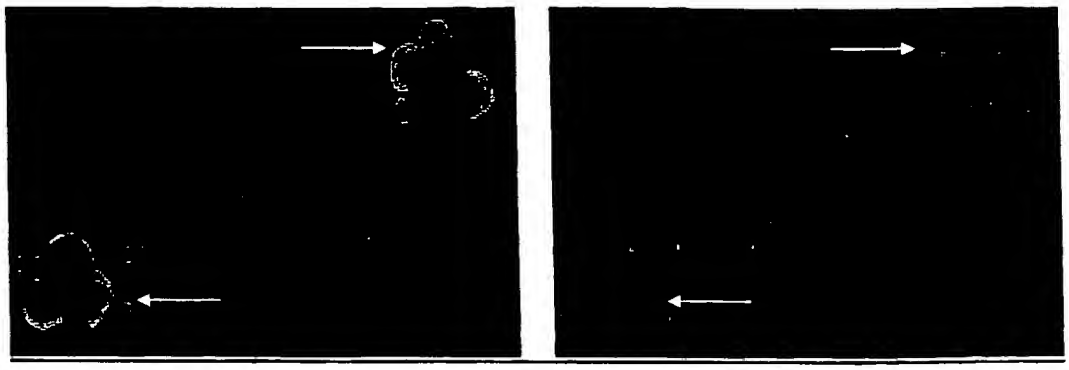

Figure 13

Figure 14-a: Sequence of wild-type Beluga LC/E (SEQ

```
     P   K   I   N   S   F   N   Y   N   D   P   V   N   D   R   T
1    CCA AAAATTAAT AGTTTTAAT TATAATGAT CCTGTTAAT GATAGAACA
     GGT TTTTAATTA TCAAAATTA ATATTACTA GGACAATTA CTATCTTGT
     I   L   Y   I   K   P   G   G   C   Q   E   F   Y   K   S
49   ATTTTATAT ATTAAACCA GGCGGTTGT CAAGAATTT TATAAATCA
     TAAAATATA TAATTTGGT CCGCCAACA GTTCTTAAA ATATTTAGT
     F   N   I   M   K   N   I   W   I   I   P   E   R   N   V
94   TTTAATATT ATGAAAAAT ATTTGGATA ATTCCAGAG AGAAATGTA
     AAATTATAA TACTTTTTA TAAACCTAT TAAGGTCTC TCTTTACAT
     I   G   T   T   P   Q   D   F   H   P   P   T   S   L   K
139  ATTGGTACA ACCCCCCAA GATTTTCAT CCGCCTACT TCATTAAAA
     TAACCATGT TGGGGGGTT CTAAAAGTA GGCGGATGA AGTAATTTT
     N   G   D   S   S   Y   Y   D   P   N   Y   L   Q   S   D
184  AATGGAGAT AGTAGTTAT TATGACCCT AATTATTTA CAAAGTGAT
     TTACCTCTA TCATCAATA ATACTGGGA TTAATAAAT GTTTCACTA
     E   E   K   D   R   F   L   K   I   V   T   K   I   F   N
229  GAAGAAAAG GATAGATTT TTAAAAATA GTCACAAAA ATATTTAAT
     CTTCTTTTC CTATCTAAA AATTTTTAT CAGTGTTTT TATAAATTA
     R   I   N   N   N   L   S   G   G   I   L   L   E   E   L
274  AGAATAAAT AATAATCTT TCAGGAGGG ATTTTATTA GAAGAACTG
     TCTTATTTA TTATTAGAA AGTCCTCCC TAAAATAAT CTTCTTGAC
     S   K   A   N   P   Y   L   G   N   D   N   T   P   D   N
319  TCAAAAGCT AATCCATAT TTAGGGAAT GATAATACT CCAGATAAT
     AGTTTTCGA TTAGGTATA AATCCCTTA CTATTATGA GGTCTATTA
     Q   F   H   I   G   D   A   S   A   V   E   I   K   F   S
364  CAATTCCAT ATTGGTGAT GCATCAGCA GTTGAGATT AAATTCTCA
     GTTAAGGTA TAACCACTA CGTAGTCGT CAACTCTAA TTTAAGAGT
     N   G   S   Q   D   I   L   L   P   N   V   I   I   M   G
409  AATGGTAGC CAAGACATA CTATTACCT AATGTTATT ATAATGGGA
     TTACCATCG GTTCTGTAT GATAATGGA TTACAATAA TATTACCCT
     A   E   P   D   L   F   E   T   N   S   S   N   I   S   L
454  GCAGAGCCT GATTTATTT GAAACTAAC AGTTCCAAT ATTTCTCTA
     CGTCTCGGA CTAAATAAA CTTTGATTG TCAAGGTTA TAAAGAGAT
     R   N   N   Y   M   P   S   N   H   G   F   G   S   I   A
499  AGAAATAAT TATATGCCA AGCAATCAC GGTTTTGGA TCAATAGCT
     TCTTTATTA ATATACGGT TCGTTAGTG CCAAAACCT AGTTATCGA
     I   V   T   F   S   P   E   Y   S   F   R   F   N   D   N
544  ATAGTAACA TTCTCACCT GAATATTCT TTTAGATTT AATGATAAT
     TATCATTGT AAGAGTGGA CTTATAAGA AAATCTAAA TTACTATTA
     S   M   N   E   F   I   Q   D   P   A   L   T   L   M   H
589  AGTATGAAT GAATTTATT CAAGATCCT GCTCTTACA TTAATGCAT
     TCATACTTA CTTAAATAA GTTCTAGGA CGAGAATGT AATTACGTA
     E   L   I   H   S   L   H   G   L   Y   G   A   K   G   I
634  GAATTAATA CATTCATTA CATGGACTA TATGGGGCT AAAGGGATT
     CTTAATTAT GTAAGTAAT GTACCTGAT ATACCCCGA TTTCCCTAA
```

Figure 14-b

```
    T  T  K    Y  T  I    T  Q  K    Q  N  P    L  I  T
679 ACTACAAAG TATACTATA ACACAAAAA CAAAATCCC CTAATAACA
    TGATGTTTC ATATGATAT TGTGTTTTT GTTTTAGGG GATTATTGT
     N  I  R    G  T  N    I  E  E    F  L  T    F  G  G
724 AATATAAGA GGTACAAAT ATTGAAGAA TTCTTAACT TTTGGAGGT
    TTATATTCT CCATGTTTA TAACTTCTT AAGAATTGA AAACCTCCA
     T  D  L    N  I  I    T  S  A    Q  S  N    D  I  Y
769 ACTGATTTA AACATTATT ACTAGTGCT CAGTCCAAT GATATCTAT
    TGACTAAAT TTGTAATAA TGATCACGA GTCAGGTTA CTATAGATA
     T  N  L    L  A  D    Y  K  K    I  A  S    K  L  S
814 ACTAATCTT CTAGCTGAT TATAAAAAA ATAGCGTCT AAACTTAGC
    TGATTAGAA GATCGACTA ATATTTTTT TATCGCAGA TTTGAATCG
     K  V  Q    V  S  N    P  L  L    N  P  Y    K  D  V
859 AAAGTACAA GTATCTAAT CCACTACTT AATCCTTAT AAAGATGTT
    TTTCATGTT CATAGATTA GGTGATGAA TTAGGAATA TTTCTACAA
     F  E  A    K  Y  G    L  D  K    D  A  S    G  I  Y
904 TTTGAAGCA AAGTATGGA TTAGATAAA GATGCTAGC GGAATTTAT
    AAACTTCGT TTCATACCT AATCTATTT CTACGATCG CCTTAAATA
     S  V  N    I  N  K    F  N  D    I  F  K    K  L  Y
949 TCGGTAAAT ATAAACAAA TTTAATGAT ATTTTTAAA AAATTATAC
    AGCCATTTA TATTTGTTT AAATTACTA TAAAAATTT TTTAATATG
     S  F  T    E  F  D    L  A  T    K  F  Q    V  K  C
994 AGCTTTACG GAATTTGAT TTAGCAACT AAATTTCAA GTTAAATGT
    TCGAAATGC CTTAAACTA AATCGTTGA TTTAAAGTT CAATTTACA
     R  Q  T    Y  I  G    Q  Y  K    Y  F  K    L  S  N
1039 AGGCAAACT TATATTGGA CAGTATAAA TACTTCAAA CTTTCAAAC
    TCCGTTTGA ATATAACCT GTCATATTT ATGAAGTTT GAAAGTTTG
     L  L  N    D  S  I    Y  N  I    S  E  G    Y  N  I
1084 TTGTTAAAT GATTCTATT TATAATATA TCAGAAGGC TATAATATA
    AACAATTTA CTAAGATAA ATATTATAT AGTCTTCCG ATATTATAT
     N  N  L    K  V  N    F  R  G    Q  N  A    N  L  N
1129 AATAATTTA AAGGTAAAT TTTAGAGGA CAGAATGCA AATTTAAAT
    TTATTAAAT TTCCATTTA AAATCTCCT GTCTTACGT TTAAATTTA
     P  R  I    I  T  P    I  T  G    R  G  L    V  K  K
1174 CCTAGAATT ATTACACCA ATTACAGGT AGAGGACTA GTAAAAAAA
    GGATCTTAA TAATGTGGT TAATGTCCA TCTCCTGAT CATTTTTTT
     I  I  R    F  C  K    N  I  V    S  V  K    G  I  R
1219 ATCATTAGA TTTTGTAAA AATATTGTT TCTGTAAAA GGCATAAGG
    TAGTAATCT AAAACATTT TTATAACAA AGACATTTT CCGTATTCC
     K  L  R
1264 AAGCTTCGC
    TTCGAAGCG
```

Figure 15-a: Sequence of chimera LC/E with N-termin...
(SEQ ID NO: 138/139)

```
        P  F  V    N  K  Q    F  N  Y    N  D  P    V  N  D
   1 CCATTTGTT AATAAACAG TTTAATTAT AATGATCCT GTTAATGAT
     GGTAAACAA TTATTTGTC AAATTAATA TTACTAGGA CAATTACTA
        R  T  I    L  Y  I    K  P  G    G  C  Q    E  F  Y
  46 AGAACAATT TTATATATT AAACCAGGC GGTTGTCAA GAATTTTAT
     TCTTGTTAA AATATATAA TTTGGTCCG CCAACAGTT CTTAAAATA
        K  S  F    N  I  M    K  N  I    W  I  I    P  E  R
  91 AAATCATTT AATATTATG AAAAATATT TGGATAATT CCAGAGAGA
     TTTAGTAAA TTATAATAC TTTTTATAA ACCTATTAA GGTCTCTCT
        N  V  I    G  T  T    P  Q  D    F  H  P    P  T  S
 136 AATGTAATT GGTACAACC CCCCAAGAT TTTCATCCG CCTACTTCA
     TTACATTAA CCATGTTGG GGGGTTCTA AAAGTAGGC GGATGAAGT
        L  K  N    G  D  S    S  Y  Y    D  P  N    Y  L  Q
 181 TTAAAAAAT GGAGATAGT AGTTATTAT GACCCTAAT TATTTACAA
     AATTTTTTA CCTCTATCA TCAATAATA CTGGGATTA ATAAATGTT
        S  D  E    E  K  D    R  F  L    K  I  V    T  K  I
 226 AGTGATGAA GAAAAGGAT AGATTTTTA AAAATAGTC ACAAAAATA
     TCACTACTT CTTTTCCTA TCTAAAAAT TTTTATCAG TGTTTTTAT
        F  N  R    I  N  N    N  L  S    G  G  I    L  L  E
 271 TTTAATAGA ATAAATAAT AATCTTTCA GGAGGGATT TTATTAGAA
     AAATTATCT TATTTATTA TTAGAAAGT CCTCCCTAA AATAATCTT
        E  L  S    K  A  N    P  Y  L    G  N  D    N  T  P
 316 GAACTGTCA AAAGCTAAT CCATATTTA GGGAATGAT AATACTCCA
     CTTGACAGT TTTCGATTA GGTATAAAT CCCTTACTA TTATGAGGT
        D  N  Q    F  H  I    G  D  A    S  A  V    E  I  K
 361 GATAATCAA TTCCATATT GGTGATGCA TCAGCAGTT GAGATTAAA
     CTATTAGTT AAGGTATAA CCACTACGT AGTCGTCAA CTCTAATTT
        F  S  N    G  S  Q    D  I  L    L  P  N    V  I  I
 406 TTCTCAAAT GGTAGCCAA GACATACTA TTACCTAAT GTTATTATA
     AAGAGTTTA CCATCGGTT CTGTATGAT AATGGATTA CAATAATAT
        M  G  A    E  P  D    L  F  E    T  N  S    S  N  I
 451 ATGGGAGCA GAGCCTGAT TTATTTGAA ACTAACAGT TCCAATATT
     TACCCTCGT CTCGGACTA AATAAACTT TGATTGTCA AGGTTATAA
        S  L  R    N  N  Y    M  P  S    N  H  G    F  G  S
 496 TCTCTAAGA AATAATTAT ATGCCAAGC AATCACGGT TTTGGAT
     AGAGATTCT TTATTAATA TACGGTTCG TTAGTGCCA AAACCTA
        I  A  I    V  T  F    S  P  E    Y  S  F    R  F
 541 ATAGCTATA GTAACATTC TCACCTGAA TATTCTTTT AGATTTA
     TATCGATAT CATTGTAAG AGTGGACTT ATAAGAAAA TCTAAA
        D  N  S    M  N  E    F  I  Q    D  P  A    L  T
 586 GATAATAGT ATGAATGAA TTTATTCAA GATCCTGCT CTTACA
     CTATTATCA TACTTACTT AAATAAGTT CTAGGACGA GAATGT
        M  H  E    L  I  H    S  L  H    G  L  Y    G  A
 631 ATGCATGAA TTAATACAT TCATTACAT GGACTATAT GGGGCT
     TACGTACTT AATTATGTA AGTAATGTA CCTGATATA CCCCGA
```

Figure 15-b

```
      G  I  T    T  K  Y    T  I  T    Q  K  Q    N  P  L
676   GGGATTACT ACAAAGTAT ACTATAACA CAAAAACAA AATCCCCTA
      CCCTAATGA TGTTTCATA TGATATTGT GTTTTTGTT TTAGGGGAT
      I  T  N    I  R  G    T  N  I    E  E  F    L  T  F
721   ATAACAAAT ATAAGAGGT ACAAATATT GAAGAATTC TTAACTTTT
      TATTGTTTA TATTCTCCA TGTTTATAA CTTCTTAAG AATTGAAAA
      G  G  T    D  L  N    I  I  T    S  A  Q    S  N  D
766   GGAGGTACT GATTTAAAC ATTATTACT AGTGCTCAG TCCAATGAT
      CCTCCATGA CTAAATTTG TAATAATGA TCACGAGTC AGGTTACTA
      I  Y  T    N  L  L    A  D  Y    K  K  I    A  S  K
811   ATCTATACT AATCTTCTA GCTGATTAT AAAAAAATA GCGTCTAAA
      TAGATATGA TTAGAAGAT CGACTAATA TTTTTTTAT CGCAGATTT
      L  S  K    V  Q  V    S  N  P    L  L  N    P  Y  K
856   CTTAGCAAA GTACAAGTA TCTAATCCA CTACTTAAT CCTTATAAA
      GAATCGTTT CATGTTCAT AGATTAGGT GATGAATTA GGAATATTT
      D  V  F    E  A  K    Y  G  L    D  K  D    A  S  G
901   GATGTTTTT GAAGCAAAG TATGGATTA GATAAAGAT GCTAGCGGA
      CTACAAAAA CTTCGTTTC ATACCTAAT CTATTTCTA CGATCGCCT
      I  Y  S    V  N  I    N  K  F    N  D  I    F  K  K
946   ATTTATTCG GTAAATATA AACAAATTT AATGATATT TTTAAAAAA
      TAAATAAGC CATTTATAT TTGTTTAAA TTACTATAA AAATTTTTT
      L  Y  S    F  T  E    F  D  L    A  T  K    F  Q  V
991   TTATACAGC TTTACGGAA TTTGATTTA GCAACTAAA TTTCAAGTT
      AATATGTCG AAATGCCTT AAACTAAAT CGTTGATTT AAAGTTCAA
      K  C  R    Q  T  Y    I  G  Q    Y  K  Y    F  K  L
1036  AAATGTAGG CAAACTTAT ATTGGACAG TATAAATAC TTCAAACTT
      TTTACATCC GTTTGAATA TAACCTGTC ATATTTATG AAGTTTGAA
      S  N  L    L  N  D    S  I  Y    N  I  S    E  G  Y
1081  TCAAACTTG TTAAATGAT TCTATTTAT AATATATCA GAAGGCTAT
      AGTTTGAAC AATTTACTA AGATAAATA TTATATAGT CTTCCGATA
      N  I  N    N  L  K    V  N  F    R  G  Q    N  A  N
1126  AATATAAAT AATTTAAAG GTAAATTTT AGAGGACAG AATGCAAAT
      TTATATTTA TTAAATTTC CATTTAAAA TCTCCTGTC TTACGTTTA
      L  N  P    R  I  I    T  P  I    T  G  R    G  L  T
1171  TTAAATCCT AGAATTATT ACACCAATT ACAGGTAGA GGACTAGTA
      AATTTAGGA TCTTAATAA TGTGGTTAA TGTCCATCT CCTGATCAT
      K  K  I    I  R  F    C  K  N    I  V  S    V  K  -
1216  AAAAAAATC ATTAGATTT TGTAAAAAT ATTGTTTCT GTAAAAC
      TTTTTTTAG TAATCTAAA ACATTTTTA TAACAAAGA CATTTTC
      I  R  K    L  R
1261  ATAAGGAAG CTTCGC
      TATTCCTTC GAAGCG
```

Figure 16-a: Sequence of chimera LC/E with LC/A di-terminus (SEQ ID NO: 140/141)

```
      P   K   I   N   S   F   N   Y   N   D   P   V   N   D   R   T
1     CCA AAAATTAAT AGTTTTAAT TATAATGAT CCTGTTAAT GATAGAACA
      GGT TTTTAATTA TCAAAATTA ATATTACTA GGACAATTA CTATCTTGT
        I   L   Y   I   K   P   G   G   C   Q   E   F   Y   K   S
49     ATTTTATAT ATTAAACCA GGCGGTTGT CAAGAATTT TATAAATCA
       TAAAATATA TAATTTGGT CCGCCAACA GTTCTTAAA ATATTTAGT
        F   N   I   M   K   N   I   W   I   I   P   E   R   N   V
94     TTTAATATT ATGAAAAAT ATTTGGATA ATTCCAGAG AGAAATGTA
       AAATTATAA TACTTTTTA TAAACCTAT TAAGGTCTC TCTTTACAT
        I   G   T   T   P   Q   D   F   H   P   P   T   S   L   K
139    ATTGGTACA ACCCCCCAA GATTTTCAT CCGCCTACT TCATTAAAA
       TAACCATGT TGGGGGGTT CTAAAAGTA GGCGGATGA AGTAATTTT
        N   G   D   S   S   Y   Y   D   P   N   Y   L   Q   S   D
184    AATGGAGAT AGTAGTTAT TATGACCCT AATTATTTA CAAAGTGAT
       TTACCTCTA TCATCAATA ATACTGGGA TTAATAAAT GTTTCACTA
        E   E   K   D   R   F   L   K   I   V   T   K   I   F   N
229    GAAGAAAAG GATAGATTT TTAAAAATA GTCACAAAA ATATTTAAT
       CTTCTTTTC CTATCTAAA AATTTTTAT CAGTGTTTT TATAAATTA
        R   I   N   N   N   L   S   G   G   I   L   L   E   E   L
274    AGAATAAAT AATAATCTT TCAGGAGGG ATTTTATTA GAAGAACTG
       TCTTATTTA TTATTAGAA AGTCCTCCC TAAAATAAT CTTCTTGAC
        S   K   A   N   P   Y   L   G   N   D   N   T   P   D   N
319    TCAAAAGCT AATCCATAT TTAGGGAAT GATAATACT CCAGATAAT
       AGTTTTCGA TTAGGTATA AATCCCTTA CTATTATGA GGTCTATTA
        Q   F   H   I   G   D   A   S   A   V   E   I   K   F   S
364    CAATTCCAT ATTGGTGAT GCATCAGCA GTTGAGATT AAATTCTCA
       GTTAAGGTA TAACCACTA CGTAGTCGT CAACTCTAA TTTAAGAGT
        N   G   S   Q   D   I   L   L   P   N   V   I   I   M   G
409    AATGGTAGC CAAGACATA CTATTACCT AATGTTATT ATAATGGGA
       TTACCATCG GTTCTGTAT GATAATGGA TTACAATAA TATTACCCT
        A   E   P   D   L   F   E   T   N   S   S   N   I   S   L
454    GCAGAGCCT GATTTATTT GAAACTAAC AGTTCCAAT ATTTCTCTA
       CGTCTCGGA CTAAATAAA CTTTGATTG TCAAGGTTA TAAAGAGAT
        R   N   N   Y   M   P   S   N   H   G   F   G   S   I   A
499    AGAAATAAT TATATGCCA AGCAATCAC GGTTTTGGA TCAATAGCT
       TCTTTATTA ATATACGGT TCGTTAGTG CCAAAACCT AGTTATCGA
        I   V   T   F   S   P   E   Y   S   F   R   F   N   D   N
544    ATAGTAACA TTCTCACCT GAATATTCT TTTAGATTT AATGATAA
       TATCATTGT AAGAGTGGA CTTATAAGA AAATCTAAA TTACTATT
        S   M   N   E   F   I   Q   D   P   A   L   T   L   M   K
589    AGTATGAAT GAATTTATT CAAGATCCT GCTCTTACA TTAATGCA
       TCATACTTA CTTAAATAA GTTCTAGGA CGAGAATGT AATTACG
        E   L   I   H   S   L   H   G   L   Y   G   A   K   G
634    GAATTAATA CATTCATTA CATGGACTA TATGGGGCT AAAGGGA
```

Figure 16-b

```
      CTTAATTAT GTAAGTAAT GTACCTGAT ATACCCCGA TTTCCCTAA
       T  T  K   Y  T  I   T  Q  K   Q  N  P   L  I  T
679   ACTACAAAG TATACTATA ACACAAAAA CAAAATCCC CTAATAACA
      TGATGTTTC ATATGATAT TGTGTTTTT GTTTTAGGG GATTATTGT
       N  I  R   G  T  N   I  E  E   F  L  T   F  G  G
724   AATATAAGA GGTACAAAT ATTGAAGAA TTCTTAACT TTTGGAGGT
      TTATATTCT CCATGTTTA TAACTTCTT AAGAATTGA AAACCTCCA
       T  D  L   N  I  I   T  S  A   Q  S  N   D  I  Y
769   ACTGATTTA AACATTATT ACTAGTGCT CAGTCCAAT GATATCTAT
      TGACTAAAT TTGTAATAA TGATCACGA GTCAGGTTA CTATAGATA
       T  N  L   L  A  D   Y  K  K   I  A  S   K  L  S
814   ACTAATCTT CTAGCTGAT TATAAAAAA ATAGCGTCT AAACTTAGC
      TGATTAGAA GATCGACTA ATATTTTTT TATCGCAGA TTTGAATCG
       K  V  Q   V  S  N   P  L  L   N  P  Y   K  D  V
859   AAAGTACAA GTATCTAAT CCACTACTT AATCCTTAT AAAGATGTT
      TTTCATGTT CATAGATTA GGTGATGAA TTAGGAATA TTTCTACAA
       F  E  A   K  Y  G   L  D  K   D  A  S   G  I  Y
904   TTTGAAGCA AAGTATGGA TTAGATAAA GATGCTAGC GGAATTTAT
      AAACTTCGT TTCATACCT AATCTATTT CTACGATCG CCTTAAATA
       S  V  N   I  N  K   F  N  D   I  F  K   K  L  Y
949   TCGGTAAAT ATAAACAAA TTTAATGAT ATTTTTAAA AAATTATAC
      AGCCATTTA TATTTGTTT AAATTACTA TAAAAATTT TTTAATATG
       S  F  T   E  F  D   L  A  T   K  F  Q   V  K  C
994   AGCTTTACG GAATTTGAT TTAGCAACT AAATTTCAA GTTAAATGT
      TCGAAATGC CTTAAACTA AATCGTTGA TTTAAAGTT CAATTTACA
       R  Q  T   Y  I  G   Q  Y  K   Y  F  K   L  S  N
1039  AGGCAAACT TATATTGGA CAGTATAAA TACTTCAAA CTTTCAAAC
      TCCGTTTGA ATATAACCT GTCATATTT ATGAAGTTT GAAAGTTTG
       L  L  N   D  S  I   Y  N  I   S  E  G   Y  N  I
1084  TTGTTAAAT GATTCTATT TATAATATA TCAGAAGGC TATAATATA
      AACAATTTA CTAAGATAA ATATTATAT AGTCTTCCG ATATTATAT
       N  N  L   K  V  N   F  R  G   Q  N  A   N  L  N
1129  AATAATTTA AAGGTAAAT TTTAGAGGA CAGAATGCA AATTTAAAT
      TTATTAAAT TTCCATTTA AAATCTCCT GTCTTACGT TTAAATTTA
       P  R  I   I  T  P   I  T  G   R  G  E   V  K  K
1174  CCTAGAATT ATTACACCA ATTACAGGT AGAGGAGAA GTAAAAAA
      GGATCTTAA TAATGTGGT TAATGTCCA TCTCCTCTT CATTTTTT
       L  L  R   F  C  K   N  I  V   S  V  K   G  I  R
1219  CTCCTTAGA TTTTGTAAA AATATTGTT TCTGTAAAA GGCATAAG
      GAGGAATCT AAAACATTT TTATAACAA AGACATTTT CCGTATTC
        K  L  R
1265  AAGCTTCGC
      TTCGAAGCG
```

Figure 17-a: Sequence of chimera LC/E with LC/A N-terminus and C-terminal di-leucine motif (SEQ ID NO: 142/143)

```
      P  F  V    N  K  Q    F  N  Y    N  D  P    V  N  D
  1   CCATTTGTT  AATAAACAG  TTTAATTAT  AATGATCCT  GTTAATGAT
      GGTAAACAA  TTATTTGTC  AAATTAATA  TTACTAGGA  CAATTACTA
      R  T  I    L  Y  I    K  P  G    G  C  Q    E  F  Y
 46   AGAACAATT  TTATATATT  AAACCAGGC  GGTTGTCAA  GAATTTTAT
      TCTTGTTAA  AATATATAA  TTTGGTCCG  CCAACAGTT  CTTAAAATA
      K  S  F    N  I  M    K  N  I    W  I  I    P  E  R
 91   AAATCATTT  AATATTATG  AAAAATATT  TGGATAATT  CCAGAGAGA
      TTTAGTAAA  TTATAATAC  TTTTTATAA  ACCTATTAA  GGTCTCTCT
      N  V  I    G  T  T    P  Q  D    F  H  P    P  T  S
136   AATGTAATT  GGTACAACC  CCCCAAGAT  TTTCATCCG  CCTACTTCA
      TTACATTAA  CCATGTTGG  GGGGTTCTA  AAAGTAGGC  GGATGAAGT
      L  K  N    G  D  S    S  Y  Y    D  P  N    Y  L  Q
181   TTAAAAAAT  GGAGATAGT  AGTTATTAT  GACCCTAAT  TATTTACAA
      AATTTTTTA  CCTCTATCA  TCAATAATA  CTGGGATTA  ATAAATGTT
      S  D  E    E  K  D    R  F  L    K  I  V    T  K  I
226   AGTGATGAA  GAAAAGGAT  AGATTTTTA  AAAATAGTC  ACAAAAATA
      TCACTACTT  CTTTTCCTA  TCTAAAAAT  TTTTATCAG  TGTTTTTAT
      F  N  R    I  N  N    N  L  S    G  G  I    L  L  E
271   TTTAATAGA  ATAAATAAT  AATCTTTCA  GGAGGGATT  TTATTAGAA
      AAATTATCT  TATTTATTA  TTAGAAAGT  CCTCCCTAA  AATAATCTT
      E  L  S    K  A  N    P  Y  L    G  N  D    N  T  P
316   GAACTGTCA  AAAGCTAAT  CCATATTTA  GGGAATGAT  AATACTCCA
      CTTGACAGT  TTTCGATTA  GGTATAAAT  CCCTTACTA  TTATGAGGT
      D  N  Q    F  H  I    G  D  A    S  A  V    E  I  K
361   GATAATCAA  TTCCATATT  GGTGATGCA  TCAGCAGTT  GAGATTAAA
      CTATTAGTT  AAGGTATAA  CCACTACGT  AGTCGTCAA  CTCTAATTT
      F  S  N    G  S  Q    D  I  L    L  P  N    V  I  I
406   TTCTCAAAT  GGTAGCCAA  GACATACTA  TTACCTAAT  GTTATTATA
      AAGAGTTTA  CCATCGGTT  CTGTATGAT  AATGGATTA  CAATAATAT
      M  G  A    E  P  D    L  F  E    T  N  S    S  N  I
451   ATGGGAGCA  GAGCCTGAT  TTATTTGAA  ACTAACAGT  TCCAATATT
      TACCCTCGT  CTCGGACTA  AATAAACTT  TGATTGTCA  AGGTTATAA
      S  L  R    N  N  Y    M  P  S    N  H  G    F  G  I
496   TCTCTAAGA  AATAATTAT  ATGCCAAGC  AATCACGGT  TTTGGAT
      AGAGATTCT  TTATTAATA  TACGGTTCG  TTAGTGCCA  AAACCTA
      I  A  I    V  T  F    S  P  E    Y  S  F    R  F
541   ATAGCTATA  GTAACATTC  TCACCTGAA  TATTCTTTT  AGATTT
      TATCGATAT  CATTGTAAG  AGTGGACTT  ATAAGAAAA  TCTAAA
      D  N  S    M  N  E    F  I  Q    D  P  A    L  T
586   GATAATAGT  ATGAATGAA  TTTATTCAA  GATCCTGCT  CTTACA
      CTATTATCA  TACTTACTT  AAATAAGTT  CTAGGACGA  GAATGT
      M  H  E    L  I  H    S  L  H    G  L  Y    G  A
631   ATGCATGAA  TTAATACAT  TCATTACAT  GGACTATAT  GGGGC
```

Figure 17-b

```
      TACGTACTT AATTATGTA AGTAATGTA CCTGATATA CCCCGATTT
       G  I  T   T  K  Y   T  I  T   Q  K  Q   N  P  L
676   GGGATTACT ACAAAGTAT ACTATAACA CAAAAACAA AATCCCCTA
      CCCTAATGA TGTTTCATA TGATATTGT GTTTTTGTT TTAGGGGAT
       I  T  N   I  R  G   T  N  I   E  E  F   L  T  F
721   ATAACAAAT ATAAGAGGT ACAAATATT GAAGAATTC TTAACTTTT
      TATTGTTTA TATTCTCCA TGTTTATAA CTTCTTAAG AATTGAAAA
       G  G  T   D  L  N   I  I  T   S  A  Q   S  N  D
766   GGAGGTACT GATTTAAAC ATTATTACT AGTGCTCAG TCCAATGAT
      CCTCCATGA CTAAATTTG TAATAATGA TCACGAGTC AGGTTACTA
       I  Y  T   N  L  L   A  D  Y   K  K  I   A  S  K
811   ATCTATACT AATCTTCTA GCTGATTAT AAAAAAATA GCGTCTAAA
      TAGATATGA TTAGAAGAT CGACTAATA TTTTTTTAT CGCAGATTT
       L  S  K   V  Q  V   S  N  P   L  L  N   P  Y  K
856   CTTAGCAAA GTACAAGTA TCTAATCCA CTACTTAAT CCTTATAAA
      GAATCGTTT CATGTTCAT AGATTAGGT GATGAATTA GGAATATTT
       D  V  F   E  A  K   Y  G  L   D  K  D   A  S  G
901   GATGTTTTT GAAGCAAAG TATGGATTA GATAAAGAT GCTAGCGGA
      CTACAAAAA CTTCGTTTC ATACCTAAT CTATTTCTA CGATCGCCT
       I  Y  S   V  N  I   N  K  F   N  D  I   F  K  K
946   ATTTATTCG GTAAATATA AACAAATTT AATGATATT TTTAAAAAA
      TAAATAAGC CATTTATAT TTGTTTAAA TTACTATAA AAATTTTTT
       L  Y  S   F  T  E   F  D  L   A  T  K   F  Q  V
991   TTATACAGC TTTACGGAA TTTGATTTA GCAACTAAA TTTCAAGTT
      AATATGTCG AAATGCCTT AAACTAAAT CGTTGATTT AAAGTTCAA
       K  C  R   Q  T  Y   I  G  Q   Y  K  Y   F  K  L
1036  AAATGTAGG CAAACTTAT ATTGGACAG TATAAATAC TTCAAACTT
      TTTACATCC GTTTGAATA TAACCTGTC ATATTTATG AAGTTTGAA
       S  N  L   L  N  D   S  I  Y   N  I  S   E  G  Y
1081  TCAAACTTG TTAAATGAT TCTATTTAT AATATATCA GAAGGCTAT
      AGTTTGAAC AATTTACTA AGATAAATA TTATATAGT CTTCCGATA
       N  I  N   N  L  K   V  N  F   R  G  Q   N  A  I
1126  AATATAAAT AATTTAAAG GTAAATTTT AGAGGACAG AATGCAAAT
      TTATATTTA TTAAATTTC CATTTAAAA TCTCCTGTC TTACGTTTA
       L  N  P   R  I  I   T  P  I   T  G  R   G  E  I
1171  TTAAATCCT AGAATTATT ACACCAATT ACAGGTAGA GGAGAAGTA
      AATTTAGGA TCTTAATAA TGTGGTTAA TGTCCATCT CCTCTTCAT
       K  K  L   L  R  F   C  K  N   I  V  S   V  K  I
1216  AAAAAACTC CTTAGATTT TGTAAAAAT ATTGTTTCT GTAAAAC
      TTTTTTGAG GAATCTAAA ACATTTTTA TAACAAAGA CATTTT
       I  R  K   L  R
1261  ATAAGGAAG CTTCGC
      TATTCCTTC GAAGCG
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